(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 560 502 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
30.10.2019 Bulletin 2019/44

(51) Int Cl.:
A61K 31/712 *(2006.01)*

(21) Application number: **19163664.6**

(22) Date of filing: **11.04.2014**

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

(30) Priority: **12.04.2013 US 201361853820 P**

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
14783392.5 / 2 983 676

(71) Applicant: **The Curators of the University of Missouri**
Columbia, MO 65211 (US)

(72) Inventors:
- **LORSON, Christian**
  Columbia,MO 65211 (US)
- **OSMAN, Erkan**
  Columbia,MO 65211 (US)

(74) Representative: **Lavoix**
Bayerstrasse 83
80335 München (DE)

Remarks:
•This application was filed on 19-03-2019 as a divisional application to the application mentioned under INID code 62.
•Claims filed after the date of the application (Rule 68(4) EPC).

(54) **SMN2 ELEMENT 1 ANTISENSE COMPOSITIONS AND METHODS AND USES THEREOF**

(57) The invention provides methods and compositions for treatment of spinal muscular atrophy (SMA). In one aspect of the invention, a series of compositions comprising an antisense oligonucleotide targeting the Element 1 site on the SMN2 pre-mRNA and a Morpholino backbone is disclosed. In another aspect of the invention, a method of treating SMA patients by modulating the splicing of SMN2 pre-mRNA to increase the amount of full-length SMN is disclosed. Certain embodiments of the inventive method comprise administering an E1-targeting antisense oligonucleotide, such as Morpholino based antisense oligonucleotide, to a SMA subject.

EP 3 560 502 A1

Description

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 61/853,820, filed April 12, 2013, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with Government support under Grant number RO1 NS041584 awarded by the National Institutes of Health. The Government has certain rights in the invention.

INCORPORATION OF SEQUENCE LISTING

[0003] A Sequence Listing is contained in the file named "13UMC005_SEQ LIST-ST25.txt" which is 4,245 bytes (measured in MS-Windows) and comprising 24 nucleic acid sequences, created April 10, 2014, is electronically filed herewith and is incorporated herein by reference in its entirety.

FIELD OF INVENTION

[0004] The present invention relates to methods and compositions for treating Spinal Muscular Atrophy (SMA), more specifically to a genetic therapy based on Element 1 antisense of SMN2 and Morpholino chemistry.

BACKGROUND OF INVENTION

[0005] Spinal Muscular Atrophies are collectively the second most common autosomal recessive neurodegenerative group of disorders with an incidence of 1 in 6000 (Crawford, T.O. and Pardo, C.A., 1996) and a carrier frequency of ~1 in 35 (Feldkotter, M. et al., 2002). The diseases are caused by the loss of α-motor neurons resulting in subsequent atrophy of voluntary muscle groups leading to paralysis and eventually to premature infantile death. Genetically the types of SMA result from a homozygous loss or mutation in the telomeric copy of the *Survival Motor Neuron-1 (SMN1)* gene. All SMA patients rely on the nearly identical copy gene, *SMN2,* which produces low levels of functional SMN protein. SMN is ubiquitously expressed and is a critical factor in a variety of RNA pathways. The best characterized SMN activity is in the assembly and maturation of the spliceosomal UsnRNPs (Meister, G., et al., 2002; Pellizzoni, L., et al., 2002). Even though the *SMN2* gene is 99% identical in nucleotide sequence and is completely identical in amino acid sequence, approximately 90% of SMN2-derived transcripts are alternatively spliced and encode a truncated protein lacking the final coding exon (exon 7). This aberrant splicing event is the result of a silent, non-polymorphic C to T nucleotide transition 6 nucleotides within exon 7 (Lorson, C.L., et al., 1999; Monani, U.R., et al., 1999). *SMN2,* however, is an excellent target for therapeutic intervention.

[0006] Cis-acting negative regulatory regions that surround *SMN2* exon 7 have been identified and described (Lorson, C.L., et al., 1999; Miyaso, H., et al., 2003; Miyajima, H., et al., 2002). In particular, ISS-N1 has been a hotspot for experimental therapeutics, especially antisense oligonucleotides (ASOs). ASO molecules of various lengths and back-bone chemistries have been used to inhibit the repressor activity of ISS-N1, leading to an increase in SMN protein and significant extensions in survival in animal models of SMA. For example, one such approach is described in US Patent No. 8,110,560 B2 to Singh et al., which discloses a series of oligonucleotide reagents that effectively target the SMN2 ISS-NI site in the SMN2 pre-mRNA. US Patent No. 8,110,560 teaches that the ISS-N 1 blocking agents target the SMN2 pre-mRNA to modulate the splicing of SMN2 to include exon 7. 2'-MOE chemistry has been used by ISIS Pharmaceuticals in the development of their ASO, SMN-Rx (Hua, Y., et al., 2011; Rigo, F., et al., 2012). Similar Morpholino-based ASOs have shown excellent pre-clinical promise in severe SMA mice and are under further development.

[0007] Still, no effective treatment exists for SMA, and the complexity and expansive clinical spectrum suggests that the SMA community cannot solely rely upon a single lead compound or genetic target.

SUMMARY OF INVENTION

[0008] One aspect of the invention is drawn to a composition for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA. Such a composition comprises an antisense oligonucleotide that comprises a sequence annealing to a first region and a second region of the SMN2 pre-mRNA. The first region is flanked by certain nucleotides, that is, the first region of the SMN2 pre-mRNA is defined by or consists of the nucleotides between -134 to -90 relative to exon 7 of the SMN2 pre-mRNA. The second region is flanked by certain nucleotides, that is, the second region of the SMN2

pre-mRNA is defined by or consists of the nucleotides between - 105 to -45 relative to exon 7 of the SMN2 pre-mRNA. In certain embodiments, the antisense oligonucleotide comprises or consists of a nucleic acid sequence selected from the group consisting of SEQ ID NO: 17 (v1.11), SEQ ID NO: 16 (v1.10), SEQ ID NO: 6 (v1.00), SEQ ID NO: 7 (v1.01), SEQ ID NO: 8 (v1.02), SEQ ID NO: 9 (v1.03), SEQ ID NO: 10 (v1.04), SEQ ID NO: 11 (v1.05), SEQ ID NO: 12(v1.06), SEQ ID NO: 13(v1.07), SEQ ID NO: 14 (v1.08). SEQ ID NO: 15 (v1.09), and SEQ ID NO: 18 (v1.12).

[0009] In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and is complementary to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA. In certain embodiments, the sequence of the antisense oligonucleotide that is complementary to the second region of the SMN2 pre-mRNA is 5' of the sequence of the antisense nucleotide that is complementary to the first region of the SMN2 pre-mRNA.

[0010] In certain embodiments, the antisense oligonucleotide is not entirely complementary to the SMN2 pre-mRNA and contains one or more substitutions. For example in certain embodiments, the antisense oligonucleotide comprises:

i. a nucleotide sequence that is complementary to, except for having one or two nucleotide substitutions, at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and is complementary to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA;
ii. a nucleotide sequence that is complementary to at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and that is complementary, except for having one or two nucleotide substitutions, to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA; or
iii. a nucleotide sequence that is complementary to, except for having one or two nucleotide substitutions, at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and that is complementary, except for having one or two nucleotide substitutions, to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA.

[0011] In certain embodiments, the antisense oligonucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 17 (v1.11), SEQ ID NO: 16 (v1.10), SEQ ID NO: 6 (v1.00), SEQ ID NO: 7 (v1.01), SEQ ID NO: 11 (v1.05), SEQ ID NO: 12(v1.06), SEQ ID NO: 13(v1.07), SEQ ID NO: 14 (v1.08). SEQ ID NO: 15 (v1.09), and SEQ ID NO: 18 (v1.12).

[0012] Certain aspects of the invention are drawn to methods for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA. In certain embodiments, such a method comprises administrating to a subject a composition for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA of the invention.

[0013] Certain asects of the invention are drawn to methods for treating Spinal Muscular Atrophy (SMA) in a human SMA patient. In certain embodiments, such a method comprises the step of administrating to the patient an effective amount of a composition for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA of the invention.

[0014] Certain aspects of the invention are drawn to a composition for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA, wherein the compositions comprising an antisense oligonucleotide with a sequence annealing to a first region and a second region of the SMN2 pre-mRNA and wherein the first region consists of the nucleotides between -134 to -120 relative to exon 7 of the SMN2 pre-mRNA and the second region consists of the nucleotides between -67 to - 54 relative to exon 7 of the SMN2 pre-mRNA.

[0015] Certain aspects of the invention are drawn to methods for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA, thereby modulating the splicing pattern of the SMN2 to generate full-length (exon-7-retaining) SMN comprising the step of administrating to a subject a composition comprising an antisense nucleotide with a sequence annealing to two distinct regions flanking E1 on the SMN2 pre-mRNA, whereas the regions consist of the nucleotides between - 134 to -120 and -67 to -54 (relative to exon 7).

[0016] In one aspect of the invention, a series of compositions capable of blocking or inhibiting the repressive activity of the SMN2 splice silencing domain, Element 1 (El), is described. In certain embodiemnts, the inventive E1 antisense oligonucleotide (ASO) anneals to two distinct regions of two distinct regions of the SMN2 pre-mRNA (intron 6 sequence), and in certain embodiments, relative to exon 7 of the SMN2 pre-mRNA, the inventive ASO anneals to: -134 to -120 and -67 to -54. In certain embodiments, the backbone for the inventive E1 ASO comprises Morphonlino residues.

[0017] In another aspect of the invention, a method of treating spinal muscular atrophy (SMA) in a subject is described. In certain embodiments, the inventive method for treating SMA comprises the step of administering to a subject an E1 ASO described herein in a dose effective to enhance the level of exon 7-containing SMN2 mRNA in cells of the subject.

[0018] In any of the compositions or methods herein, the antisense oligonucleotide can comprise a Morpholino backbone.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]

FIG. 1 is a schematic illustration of the location of Element 1 relative to Exon 7 of the SMN2 pre-mRNA and an exemplary antisense oligonucleotide according to one embodiment of the invention.
FIG. 2 is a sequence alignment showing the sequences of SEQ ID NOs: 6-18 complementary to the SMN2 pre-mRNA -133 to -46 region upstream of intron 7.
FIG. 3 shows weight gains among treated mice and controls in Example 1.
FIG. 4 shows survival among treated mice and controls in Example 1.
FIG. 5 is a bar graph summarizing the weight gains among treated mice and controls in Example 2.
FIG. 6 is a bar graph summarizing the "Time to Right" motor function test among treated mice and controls in Example 2.
FIG. 7 is a graph summarizing the grip strength motor function test among treated mice and controls in Example 2.
FIG. 8 is a graph summarizing the Rota-rod performance test between treated mice and the wild controls in Example 2.
FIG. 9 is a graph summarizing the survival data among treated mice and various controls in Example 2.
FIG. 10 is an array and Western Blot analysis on protein induction among the treated mice and controls in Example 2.
FIG. 11 is a quantitative graph for the Western Blot analysis in Example 2.
FIG. 12a shows the RT-PCR analysis for SMN-full length and SMNA7 levels in the cells of treated mice and controls in Example 2.
FIG. 12b is a quantitative graph for SMN-full length and SMNA7 levels in the cells of the treated mice and controls in Example 2.
FIG. 13 illustrates targeting of the intronic repressor Element 1 with antisense oligonucleotides versus previously published ASO sequences targeting the intronic silencer ISS-N1.
FIG. 14 shows an increase in full-length SMN transcript after $E1^{MO}$-ASO treatment in Example 3.
FIG. 15 shows that injection of morpholino based ASO targeting the Element1 repressor increased total SMN protein in the A7SMA mouse model in Example 3.
FIG. 16a shows that severe SMNΔ7 SMA mice showed significant improvement in survival and longevity after injections with $E1^{MO}$-ASO oligos in Example 3.
FIG. 16b shows survival curves demonstrating a significant increase in life expectancy for $E1^{MO}$-ASO ICV, ICV&ICV and ICV&IP injected animals in Example 3.
FIG. 17 shows that $E1^{MO}$-ASO treatment results in a significant weight gain in Example 3.
FIG. 18a shows the percent weight gained from birth to peak was also compared between groups treated in Example 3.
FIG. 18b shows statistical significance between each treatment group in FIG. 18a.
FIG. 19 shows individual weights on P12 for all controls and animals treated with $E1^{MO}$-ASO in Example 3.
FIG. 20 is a bar graph showing the percent of all tested animal groups able to right themselves from a prone position in Example 3.
FIG. 21a is a graph representing raw data of the average time to right from P7 to P25 in Example 3.
FIG. 21b is a scatter plot of TTR performance of mice injected with $E1^{MO}$-ASO in Example 3.
FIG. 22 shows grip strength measurements in grams in Example 3.
FIG. 23 shows results of the rotarod performance test in Example 3.
FIG. 24a is a Western blot (n=3) showing increased SMN in spinal cord tissue of five (5) ICV injected animals with $E1^{MO}$-ASO in Example 3.
FIG. 24b shows Western blot quantification of FIG. 24a.
FIG. 25 shows that the treated SMN^{RT} animals (labeled "SMN^{RT} $E1^{MO}$-ASO") and the unaffected littermates (labeled "SMN^{RT} Unaffected") were substantially more vigorous and lived more than 180 days compared to the untreated mice (labeled "SMA^{RT} Untreated") in Example 3.
FIG. 26 shows a significant increase in the average weight of SMN^{RT} animal model after treatment with $E1^{MO}$-ASO in Example 3.
FIG. 27 show a comparison of the percent weight gained from birth to peak between animal groups in Example 3.
FIG. 28 shows individual weights on P12 in Example 3.
FIG. 29 shows the percent of the tested animals able to right themselves compared to untreated mice where the TTR reflex is delayed in Example 3.
FIG. 30 shows the average time to right from P7 to P25 for all experimental groups in Example 3.
FIG. 31 is a scatter plot of TTR performance of SMN^{RT} mice injected with $E1^{MO}$-ASO in Example 3.
FIG. 32 shows grip strength measurements in grams in Example 3.
FIG. 33 shows results of the rotarod performance test in Example 3.
FIG. 34 is RT-PCR quantification showing the percent increase in full length SMN transcript in three SMA animals after treatment with $E1^{MO}$-ASO in Example 3.
FIG. 35 is Western blot quantification showing the percent increase in SMN protein induction was compared to the unaffected and untreated control group and plotted in a bar graph (n=5) in Example 3.

DESCRIPTION OF THE SEQUENCES

[0020] Illustrative examples of sequences useful in certain embodiments of the invention, including antisense oligo sequences targeting the E1 repressor include, but are not limited to, the following:

1. SEQ ID NO: 1 is a partial Intron 6 sequence containing the entire Element 1 (-112; -67):

5′-

TGCAAGAAAACCTTAACTGCAGCCTAATAATTGTTTTCTTTGGGATAACTTTTAAAGTACAT

TAAAAGACTATCAACTTAATTTCTGATCATATTTTGTTGAATAAAATAA**GTAAAATGTCTTG**

TGAAACAAAATGCTTTTTTAACATCCATATAAAGCTATCTATATATAGCTATCTATGTCTATA

TAGCTATTTTTTTTAACTTCCTTTATTTTCCTTACA [**EXON7**]-3′ (SEQ ID NO: 1)

2. SEQ ID NO: 2 is the Miaso 45-mer Element 1 (-112; -67) within Intron 6: 5'-(112)GTAAAATGTCTTGT-GAAACAAAATGCTTTTTAACATCCATATAAA(67)-3' (SEQ ID NO: 2) (Miaso 45-mer; **bold** within SEQ ID NO: 1 above)

3. SEQ ID NO: 3 is a partial sequence of SEQ ID NO: 1 comprising E1 flanking regions shown in bold:

5′-

(134)**ATATTTTGTTGAATAAAATAA**GTAAAATGTCTTGTGAAACAAAATGCTTTTTAACAT

CCATATAAA**GCTATCTATATATAGCTATCT**(54)-3′(SEQ ID NO: 3)

4. SEQ ID NO: 4 is the reverse of SEQ ID NO: 3 (i.e, SEQ ID NO: 3 shown 3' to 5'):

3′-

TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAA

AATGAATAAAATAAGTTGTTTTATA-5′ (SEQ ID NO: 4)

5. SEQ ID NO: 5 is an ASO-based bifunctional (BiF) RNA targeting the E1 repressor: 5'-CTATATATAGATAGTTAT-TCAACAAAACTAGTAATTTTT-3' (SEQ ID NO: 5)

6. SEQ ID NO: 6 is a 26-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.00 ASO": 5'-CTATATATAGATAGTTATTCAACAAA-3' (SEQ ID NO: 6; v1. 00)

7. SEQ ID NO: 7 is a 25-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.01 ASO": 5'-TAGATAGCTTTACATTTTACTTATT-3' (SEQ ID NO: 7; v1. 01)

8. SEQ ID NO: 8 is a 25-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.02 ASO": 5'-TATGGATGTTAAAAAGCATTTTGTT-3' (SEQ ID NO: 8; v1. 02)

9. SEQ ID NO: 9 is a 25-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.03 ASO": 5'-CTATATATAGATAGCTTTATATGGA-3' (SEQ ID NO: 9; v1.03)

10. SEQ ID NO: 10 is a 25-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.04 ASO": 5'-CATTTTACTTATTTTATTCAACAAA-3' (SEQ ID NO: 10; v1.04)

11. SEQ ID NO: 11 is a 25-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.05 ASO": 5'-GCTTTATATGGACATTTTACTTATT-3' (SEQ ID NO: 11; v1.05)

12. SEQ ID NO: 12 is a 25-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.06 ASO": 5'-GATGTTAAAAAGCGTTTCACAAGAC-3' (SEQ ID NO: 12; v1.06)

13. SEQ ID NO: 13 is a 25-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.07 ASO": 5'-TATATGGATGTTATTATTCAACAAA-3' (SEQ ID NO: 13; v1.07)
14. SEQ ID NO: 14 is a 25-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.08 ASO": 5'-GCATTTTGTTTCACAAGTTATTCAA-3' (SEQ ID NO: 14; v1.08)
15. SEQ ID NO: 15 is a 25-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.09 ASO": 5'-CTATATATAGATAGCGACATTTTAC-3' (SEQ ID NO: 15; v1.09)
16. SEQ ID NO: 16 is a 26-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.10 ASO": 5'-AGATAGCTTTATATGGATTTATTCAA-3' (SEQ ID NO: 16; v1. 10)
17. SEQ ID NO: 17 is a 20-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1.11 ASO": 5'-CTATATATAGTTATTCAACA-3' (SEQ ID NO: 17; v1. 11)
18. SEQ ID NO: 18 is a 24-mer antisense sequence targeting the E1 repressor referred to herein as "Element 1 v1. 12ASO": 5'-TTTATATGGATGAAGACATTTTAC-3' (SEQ ID NO: 18; v1. 12)
19. SEQ ID NO: 19 is a mSmn-WT forward primer: 5'-tctgtgttcgtgcgtggtgacttt-3' (SEQ ID NO: 19)
20. SEQ ID NO: 20 is a mSmn-WT reverse primer: 5'-cccaccacctaagaaagcctcaat-3' (SEQ ID NO: 20)
21. SEQ ID NO: 21 is a *Smn* knockout SMN1-KO forward primer: 5'-ccaacttaatcgccttgcagcaca-3' (SEQ ID NO: 21)
22. SEQ ID NO: 22 is a Smn knockout SMN1-KO reverse primer: 5'-aagcgagtggcaacatggaaatcg-3' (SEQ ID NO: 22)
23. SEQ ID NO: 23 is a negative scrambled control: 5'-CCU CUU ACC UCA GUU ACA AUU UAU A-3' (SEQ ID NO: 23)
24. SEQ ID NO: 24 is a $E1^{MO}$-ASO(26-mer): 5'-CUA UAU AUA GAU AGU UAU UCA ACA AA-3' (SEQ ID NO: 24)

DETAILED DESCRIPTION

[0021] Spinal muscular atrophy (SMA) is a neurodegenerative disease caused by the loss of *Survival Motor Neuron-1 (SMN1)* (SMN1=survival of motor neuron 1, telomeric [*Homo sapiens*] GenBank accession number NG_008691.1 (Genomic); NC_000005.10 (Chromosome); NM_000344.3--->NP _000335.1 (mRNA & Protein)). In all SMA patients a nearly identical copy gene called *SMN2* is present which produces low levels of functional protein due to an alternative splicing event (SMN2=survival of motor neuron 2, centromeric [*Homo sapiens*] GenBank accession number NG_008728.1 (Genomic); NC_000005.10 (Chromosome); NM_017411.3->NP_059107.1 (mRNA & Protein)).

[0022] Without being bound by theory, certain aspects of the invention are drawn to preventing exon-skipping by targeting an intronic repressor, SMN2 Element 1 (E1), located upstream (5'-) of *SMN2* exon 7 (FIG. 1). In certain embodiments, E1 and/or regions upstream (5'-) and/or downstream (3'-) of E1 are targeted using compositions comprising antisense-oligonucleotides (referred to herein generally as E1-ASOs) (illustrative examples shown in FIG. 2). Certain embodiments are drawn to compositions for blocking or inhibiting the repressive activity of the Element E1 of the SMN2 pre-mRNA wherein the composition comprises an antisense oligonucleotide. It is understood that a composition comprising an antisense oligonucleotide could consist or essentially consist of an antisense oligonucleotide such that certain embodiments are drawn to antisense oligonucleotides for blocking or inhibiting the repressive activity of the Element E1 of the SMN2 pre-mRNA. As used herein, "blocking" or "inhibiting" is used to describe the process of limiting and/or preventing the repressor function of SMN2 Element 1. In certain embodiments, any of the antisense oligonucleotides described herein are Morpholino-based antisense oligonucleotides (referred to herein generally as $E1^{MO}$-ASOs). Morpholino oligonucleotides, as referred to herein, comprise morpholine rings in their backbones, which replace the ribose or deoxyribose rings characteristic of RNA and DNA oligonucleotides. Morpholinos contain uncharged phosphorodiamidate inter-subunit linkages instead of the anionic phosphodiester linkage found in natural nucleic acids. The morpholine rings carry A, C, G or T bases positioned suitably for Watson-Crick base pairing.

[0023] SMN2 Element 1 (E1) has been previously explored by characterizing the genetic region upstream of SMN2 exon 7 as a repressor of SMN2 exon 7 inclusion. The genetic activity of E1 reduces the production of the full-length SMN product by promoting the exclusion of exon 7 and the expression of the truncated isoform (SMN-delta 7). 2'-O-Methyl ASO-based bifunctional (BiF) RNAs have been tested that target the E1 repressor and with ICV injection extended survival by ~48 hours. BiF RNAs are ASO-like molecules that derive their name from the presence of two functional domains: an RNA sequence that is an antisense element complementary to a specific cellular RNA (e.g. SMN Intron 6, Exon 7, or Intron 7); and an untethered RNA segment that serves as a sequence-specific binding platform for cellular splicing factors, such as SR proteins. The 5' end of exon 7 was targeted with the antisense element; however, it is possible that an antisense sequence within exon 7 does not allow for proper recognition of the necessary splicing signals. To enhance the activity of the SMN bifunctional RNAs, a set of RNAs that targeted El and ISS-N1 were developed. By targeting a repressor sequence with the anti-sense sequence, there was a 2-fold mechanism of SMN induction: inhibition of the intronic repressor and recruitment of SR proteins via the SR recruitment sequence of the bifunctional RNA. Based upon molecular understanding of SMN exon 7 regulation, high affinity binding sites for hTra21 or SF2/ASF - two factors known to stimulate exon 7 inclusion were incorporated. However, the 2'-O-Methyl chemistry used in these experiments has proven to be suboptimal for *in vivo* activity.

[0024] Antisense oligonucleotides targeting the E1 region and/or surrounding regions of SMN2 (i.e., distinct from

targeting ISS-N1) have been developed and examined in two important animal models of disease: the "gold standard" SMNΔ7 mouse, which is a very severe model living only ~14 days; and a recently developed model called SMN^{RT}, in which animals live ~35 days and represent a less severe population. Work was done in transgenic mouse that has the human SMN2 gene. All data herein (e.g., RNA, protein, etc.) represent the human SMN2 gene in a mouse with the mouse Smn gene deleted (Smn1=survival motor neuron 1 [*Mus musculus* (house mouse)] GenBank accession number NT_187006.1 (Genomic); NC_000079.6 (Chromosome); NM_011420.2 → NP_035550.1 survival motor neuron protein isoform 1 (mRNA & Protein) NM_001252629.1 → NP_001239558.1 survival motor neuron protein isoform 2 (mRNA & Protein)). Therefore, in certain embodiments, antisense oligonucleotides are targeted to the human SMN2 gene.

[0025] It has been discovered that using a relatively low dose of certain Element 1 Morpholino ASOs ($E1^{MO}$-ASOS), the SMA phenotype at the molecular, cellular, and organismal levels were largely rescued, including a 300-700% extension in survival for the two mouse models. From a pre-clinical perspective, there is excellent target engagement (*SMN2* splicing), molecular efficacy (SMN protein production), and robust phenotypic rescue in two complementary models of disease. Collectively, this work identifies lead ASO candidates that target a distinct region of the SMN2 pre-mRNA.

[0026] Representative embodiments of the invention are directed to new methods and compositions based on Antisense Oligonucleotides (ASOs) technology and Morpholino chemistry for modulating the SMN2 splicing pattern to generate increased levels of exon 7-containing full-length SMN. In certain embodiments, the increased level of exon 7-containing full-length SMA is sufficient to provide a viable therapy to Spinal Muscular Atrophy (SMA) patients. Certain embodiments comprise a series of compositions capable of blocking or inhibiting the repressive activity of the SMN2 splice silencing domain, Element 1 (E1). For example, certain embodiments comprise E1 antisense oligonucleotide based compositions that block or inhibit the splice inhibitory effects of the E1, thereby modulating splicing of the SMN2 pre-mRNA to generate exon 7 retaining full-length SMN.

[0027] In certain embodiments, a composition comprises an E1 antisense oligonucleotide (E1-ASO) with a sequence annealing to two distinct regions flanking E1 on the SMN2 pre-mRNA, wherein the regions comprise the nucleotides between -134 to -120 and -67 to -54 (relative to exon 7). In certain embodiments, such E1-ASO further comprises a Morpholino backbone.

[0028] FIG. 1 illustrates an exemplary E1-ASO. As shown in FIG. 1, the E1-ASO is designed with two split antisense sequences annealing to two regions flanking repressor E1: Region (67-54) and Region (134-120). To increase exon 7-containing SMN expression, a two-pronged strategy to design the antisense may be used: on one side includes two antisense regions that block E1 and on the other end a sequence that recruits exonic splice enhancers specific for exon 7.

[0029] Other embodiments can incorporate additional antisense oligonucleotides annealing to the sequences on either side of the two regions-i.e., Region (-67 to -54) and Region (-134 to -120)-and/or sequences within or partially within the E1 motif. FIG. 2 is an alignment (sequences shown reversed, i.e., 3' to 5') of the region comprising the nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7, including the E1 motif (*italicized nucleotides*), and showing in **bold underline** the sequences of the region of the SMN2 pre-mRNA complementary to the antisense oligonucleotide sequences of SEQ ID NOs: 6 to 18, i.e., v1.00 to v.1.12, respectively.

[0030] Certain embodiments are drawn to compositions comprising an antisense oligonucleotide that comprises a nucleotide sequence that anneals to the region comprising nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7. In certain embodiments, the antisense oligonucleotide comprises a sequence that anneals to two regions of the nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7, wherein the first region comprises the nucleotides between -134 to -90 relative to exon 7 of the SMN2 pre-mRNA and the second region comprises the nucleotides between -105 and -45 relative to exon 7 of the SMN2 pre-mRNA. In order for an antisense oligonucleotide targeting the E1 region to modulate the activity of Element 1, it is understood that "anneal(s)" or "annealing," as used herein, refers to annealing of two substantially complementary nucleic acid molecules under physiological conditions. In certain embodiments, the first region comprises: the nucleotides between -134 to -90; the nucleotides between -134 to -95; the nucleotides between -134 to -100; the nucleotides between -134 to -105; the nucleotides between -134 to -110; or the nucleotides between -134 to -115, relative to exon 7 of the SMN2 pre-mRNA. In certain embodiments, the second region comprises: the nucleotides between -90 to -45; the nucleotides between -85 to -45; the nucleotides between -80 to -45; the nucleotides between -75 to -45; the nucleotides between -70 to -45; or the nucleotides between -65 to -45, relative to exon 7 of the SMN2 pre-mRNA. In certain embodiments, the first and/or second regions consist of any of the above defined regions upstream of exon 7 of the SMN2 pre-mRNA. In certain embodiments the first and second regions of the SMN2 pre-mRNA are a combination of any of the above first and second regions, for example the first region comprises the nucleotides between -134 to -95 and the second region comprises the nucleotides between -85 to -45, for example the first region comprises the nucleotides between -134 to -115 and the second region comprises the nucleotides between -65 to -45, etc.

[0031] In certain embodiments, the antisense oligonucleotide sequence comprises a certain number of nucleotides that are complementary to consecutive nucleotides of the region comprising the nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence

that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26 consecutive nucleotides of the region comprising nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7. In certain embodiments, the antisense oligonucleotide comprising a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26 consecutive nucleotides of the region comprising nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7 is not entirely complementary and comprises one, two, three, four, five, or six nucleotide substitutions in the antisense oligonucleotide sequence. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the first regions listed herein. In certain embodiments, the antisense oligonucleotide comprising a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the first regions listed herein is not entirely complementary and comprises one, two, three, four, five, or six, nucleotide substitutions in the antisense oligonucleotide sequence. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the second regions listed herein. In certain embodiments, the antisense oligonucleotide comprising a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the second regions listed herein is not entirely complementary and comprises one, two, three, four, five, or six, nucleotide substitutions in the antisense oligonucleotide sequence. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the first regions listed herein and complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the second regions listed herein. In certain embodiments, the antisense oligonucleotide comprising a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the first regions listed herein and complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the second regions listed herein is not entirely complementary and comprises one, two, three, four, five, or six, nucleotide substitutions in the antisense oligonucleotide sequence. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least eight consecutive nucleotides of any of the first regions listed herein. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least eight consecutive nucleotides of any of the second regions listed herein. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least eight consecutive nucleotides of any of the first regions listed herein and complementary to at least eight consecutive nucleotides of any of the second regions listed herein. In the SMN2 pre-mRNA, the first regions listed herein are upstream (5') of the second regions listed herein. In the antisense oligonucleotide sequences, however, the nucleotide sequence of the antisense oligonucleotide that is complementary to the second region of the SMN2 pre-mRNA is upstream (5') of the nucleotide sequence of the antisense oligonucleotide that is complementary to the first region of the SMN2 pre-mRNA.

[0032] Certain embodiments are drawn to compositions comprising an antisense oligonucleotide that comprises a nucleotide sequence that anneals to the region consisting of nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7. In certain embodiments, the antisense oligonucleotide comprises a sequence that anneals to two regions of the nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7, wherein the first region consists of the nucleotides between -134 to -90 relative to exon 7 of the SMN2 pre-mRNA and the second region consists of the nucleotides between -105 and -45 relative to exon 7 of the SMN2 pre-mRNA. In order for an antisense oligonucleotide targeting the E1 region to modulate the activity of Element 1, it is understood that "anneal(s)" or "annealing," as used herein, refers to annealing of two substantially complementary nucleic acid molecules under physiological conditions. In certain embodiments, the first region consists of: the nucleotides between -134 to -90; the nucleotides between -134 to -95; the nucleotides between -134 to -100; the nucleotides between -134 to -105; the nucleotides between -134 to -110; or the nucleotides between -134 to -115, relative to exon 7 of the SMN2 pre-mRNA. In certain embodiments, the second region consists of: the nucleotides between -90 to -45; the nucleotides between -85 to -45; the nucleotides between -80 to -45; the nucleotides between -75 to -45; the nucleotides between -70 to -45; or the nucleotides between -65 to -45, relative to exon 7 of the SMN2 pre-mRNA. In certain embodiments, the first and/or second regions consist of any of the above defined regions upstream of exon 7 of the SMN2 pre-mRNA. In certain embodiments the first and second regions of the SMN2 pre-mRNA are a combination of any of the above first and second regions, for example the first region consists of the nucleotides between -134 to -95 and the second region consists of the nucleotides between -85 to -45, for example the first region consists of the nucleotides between -134 to -115 and the second region consists of the nucleotides between -65 to -45, etc.

[0033] In certain embodiments, the antisense oligonucleotide sequence comprises a certain number of nucleotides that are complementary to consecutive nucleotides of the region consisting of the nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26 consecutive nucleotides of the region consisting of nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7. In certain embodiments, the antisense oligonucleotide comprising a nucleotide sequence that is complementary to at least 6, 7, 8, 9,

10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26 consecutive nucleotides of the region consisting of nucleotides -46 to -133 that are 5' (upstream) of SMN2 Exon 7 is not entirely complementary and comprises one, two, three, four, five, or six nucleotide substitutions in the antisense oligonucleotide sequence. In certain embodiments, the antisense oligonucleotide comprising a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the first regions listed herein. In certain embodiments, the antisense oligonucleotide comprising a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the first regions listed herein is not entirely complementary and comprises one, two, three, four, five, or six, nucleotide substitutions in the antisense oligonucleotide sequence. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the second regions listed herein. In certain embodiments, the antisense oligonucleotide comprising a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the second regions listed herein is not entirely complementary and comprises one, two, three, four, five, or six, nucleotide substitutions in the antisense oligonucleotide sequence. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the first regions listed herein and complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the second regions listed herein. In certain embodiments, the antisense oligonucleotide comprising a nucleotide sequence that is complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the first regions listed herein and complementary to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive nucleotides of any of the second regions listed herein is not entirely complementary and comprises one, two, three, four, five, or six, nucleotide substitutions in the antisense oligonucleotide sequence. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least eight consecutive nucleotides of any of the first regions listed herein. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least eight consecutive nucleotides of any of the second regions listed herein. In certain embodiments, the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least eight consecutive nucleotides of any of the first regions listed herein and complementary to at least eight consecutive nucleotides of any of the second regions listed herein. In the SMN2 pre-mRNA, the first regions listed herein are upstream (5') of the second regions listed herein. In the antisense oligonucleotide sequences, however, the nucleotide sequence of the antisense oligonucleotide that is complementary to the second region of the SMN2 pre-mRNA is upstream (5') of the nucleotide sequence of the antisense oligonucleotide that is complementary to the first region of the SMN2 pre-mRNA.

[0034] In certain embodiments, while the antisense oligonucleotide anneals to and/or comprises a nucleotide sequence that is complementary to a first region and a second region upstream of exon 7 of the SMN2 pre-mRNA the antisense oligonucleotide sequence is non-sequential, there is a portion of the sequence of the SMN2 pre-mRNA that intervenes between the sequences of the SMN2 pre-mRNA to which the antisense oligonucleotide anneals or is complementary to. That is, the entire sequence of the antisense oligonucleotide is not complementary to a wholly consecutive sequence of the SMN2 pre-mRNA sequence. This is illustrated in FIG. 2, where the entire sequences of v1.00, v1.01, v1.05, v1.06, v1.07, v1.08, v.1.09, v1.10, v1.11, and v1.12 (corresponding to SEQ ID NOs: 6, and 11-18), are non-sequential with respect to the SMN2 pre-mRNA, that is split by intervening sequences of the SMN2 pre-mRNA.

[0035] Certain embodiments are drawn to compositions comprising an antisense oligonucleotide wherein the antisense oligonucleotide comprises, or in certain embodiments consists of, a nucleic acid sequence of SEQ ID NO: 6 (v1.00), SEQ ID NO: 7 (v1.01), SEQ ID NO: 8 (v1.02), SEQ ID NO: 9 (v1.03), SEQ ID NO: 10 (v1.04), SEQ ID NO: 11 (v1.05), SEQ ID NO: 12 (v1.06), SEQ ID NO: 13 (v1.07), SEQ ID NO: 14 (v1.08), SEQ ID NO: 15 (v1.09), SEQ ID NO: 16 (v1.10), SEQ ID NO: 17 (v1.11), and SEQ ID NO: 18 (v1.12). In certain embodiments, the antisense oligonucleotide sequence comprises, or in certain embodiments consists of, a nucleic acid sequence comprising one, two, three, four, five, or six nucleotide substitutions in the nucleotide sequence of any of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18.

[0036] In certain embodiments, any of the antisense oligonucleotides disclosed herein can be a modified nucleotide, such as a Morpholino antisense oligonucleotide.

[0037] Certain embodiments provide for methods of blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA wherein the method comprises administrating to a subject a composition of the invention. Certain embodiments provide for methods of treating a SMA subject. In certain embodiments, the method comprises the step of administering to a subject an E1-ASO of an embodiment of the invention. The ability to block or inhibit the repressive activity of the Element 1 of the SMN2 pre-mRNA by an antisense oligonucleotide of the invention is does dependent. In certain embodiments, the E1-ASO is administered in a dose effective manner to enhance the level of exon 7-containing SMN2 mRNA in cells of the subject. In certain embodiments, the E1-ASO is administered in an effective amount to treat SMA in a patient. In certain embodiments, the patient is a mammal, such as a human. In certain embodiments, the E1-ASO is a Mopholino modified E1 ASO. One of ordinary skill in the art would understand that antisense oligonucleotides such

as those described herein, including Morpholino modified nucleic acids, are commercially synthesized and/or otherwise produced by known methods. Intracerebroventricular (ICV), intraperitoneal (IP), and intravenous (IV) administration have been shown to result in increases in SMN protein. Combinatorial injections have proven to be the most efficacious. Thus, in certain embodiments, E1 ASOs can be administered via ICV, IP, IV, or a combinatorial administration thereof into a subject. In certain embodiments, an antisense oligonucleotide of the invention is administered via a 1 μM, 2 μM, 3 μM, 4 μM, or 5 μM ICV injection. In certain methods, a doubling dose is achieved via two ICV injections or an ICV+IP dosing.

[0038] In certain embodiments, the subject is a mammal. In certain embodiments, the subject is a human. In certain embodiments, the subject is a rodent such as a mouse or rat, for example, a transgenic mouse such as strain SMAΔ7 and "readthrough" mice (severe and intermediate forms of SMA). For example, following treatment, SMA mice showed significant weight gain, ambulated at near normal levels, lived 200 to 600% longer, and exhibited near-wild type levels of full-length (exon 7 retaining) SMN protein.

[0039] The following disclosed embodiments are merely representative of the invention which may be embodied in various forms. Thus, specific structural, functional, and procedural details disclosed in the following Examples are not to be interpreted as limiting.

EXAMPLES

Example 1

[0040] FIG. 3 shows the results of weight gain for SMA mice versus controls wherein the mice were injected with Morpholino antisense oligonucleotides comprising the nucleotide sequences of SEQ ID NOs: 6-18 (E1 V.00 (original) and E1 V.01 to V.12, respectively).

[0041] FIG. 4 shows the results of survival for SMA mice versus controls wherein the mice were injected with Morpholino antisense oligonucleotides comprising the nucleotide sequences of SEQ ID NOs: 6-18 (E1 V.00 (original) and V.01 to V.12, respectively).

Example 2

[0042] FIG. 5 shows weight gains among $E1^{MO}$-ASO-v1.00-treated mice (severe form) and controls, showing the weight gained from birth to when a peak weight was reached. There were three control groups as well: 1) Animals injected with scrambled MO-ASOs (no binding specificity); 2) Unaffected healthy animals (heterozygous); and 3) Untreated SMA animals. As the data shows, IP injection had a slight improvement effect on weight gain, however, ICV injection alone had a much more significant impact on weight gain. Moreover, when a combination of both types of injections was used, the weight gain for the treated animals reaches almost 900% of their initial birth weight. Statistical calculations show the significance in the Table 1:

Table 1.

Treatment	Avg Weights	Std Deviation	Std Error	P value E1 Morph ICV	P value Untreated	P value Scrambled
Scrambled	104%	0.351	0.101	0.00001072	0.40907992	1.00000000
Untreated	115%	0.233	0.067	0.00001517	1.00000000	0.40907992
E1 Morph IP Only	197%	0.265	0.119	0.01520258	0.00001147	0.00009196
E1 Morph ICV Only	551%	3.031	0.553	1.00000000	0.00001517	0.00000964
E1 Morph ICV & IP	874%	4.412	1.103	0.00343686	0.00000061	0.00000050
Heterozygous	1380%	1.520	0.439	0.00000000	0.00000000	0.00000000

[0043] FIG. 6 shows the average times taken for animals (treated mice (severe form) and controls) to right themselves. Righting reflex is a motor function test performed on animals. Time-To-Right has been previously shown to be a sensitive measurement of gross motor function for SMA animals. In short, animals are placed on their backs and the time required to turn upright is measured. Animals that failed to turn in 30 seconds are considered failing the test. As shown in FIG. 6, measurements are from day 7 through 25. Healthy (heterozygous) animals can do this test within 1-5 seconds from day 7 onward. Although the IP injected animals failed this motor function test, the ICV and also the ICV&IP combinatorial injections have an incredible impact on the motor functions on the tested animals. After two weeks the ICV injected animals righted themselves under 15 seconds and the ICV&IP injected animals did so under 10 seconds. By day 20 the

ICV&IP injected animals were turning under 5 seconds.

[0044] FIG. 7 shows a comparison of grip strength between treated mice (severe form) and wild heterozygous mice. Grip strength is another motor function test to measure muscle functionality; the grip strength test is performed by placing animals on a device that measures the animal's pull (grip). The test compared the strength of the unaffected heterozygous mice and the MO-ASOs treated SMA animals after ICV&IP injections. 20 trials were measured on the days indicated from P25 to P91.

[0045] FIG. 8 shows another performance test comparing treated mice and wild mice. In the Roto-Rod performance test, animals are placed on a rotating axle, while time is measured for their ability to stay on without falling. In the beginning, treated animals performed exactly like their healthy littermates. With time, their strength weakens and their performance decreases. Up until around day 45, treated animals performed virtually as unaffected heterozygous animals.

[0046] FIG. 9 shows a comparison of the survival data among treated mice and controls. As shown in FIG. 9, the IP only injected animals had a very slight extension of survival. However, the ICV injected animals reached an average of 39 days (max. 83 days), whereas, the combinatorial ICV&IP injections extended the average life span to 54 days (max. 89 days). The extension of survival of the treated animals demonstrates the clinic potential of treatment based on ASOs targeting E1.

[0047] FIG. 10 shows Western-blot analysis to determine the SMN protein induction in treated animals (severe form with ICV injections only) compared to controls. Protein induction was significantly higher in all tissues tested with substantial increase in spinal cord and muscle tissues. Three separate mice were used to determine the significance in the protein induction.

[0048] FIG. 11 shows protein induction in different types of tissue. As shown in FIG. 11, three quantification graphs of the Western Blots data further confirmed the significant increase in protein production especially in muscle and spinal cord tissues. The Western Blots analysis further proved the viability of the treatment based on the inventive ASOs targeting E1.

[0049] The same experimental and testing design was been applied to a different, intermediate mouse model (the "Readthrough" mice), representing a milder form of SMA, through the ICV injections only. The data on weight gain increases, various motor functionality tests, and average protein induction analysis was comparable to that of the treated severe-form mice.

[0050] FIG. 12(a,b) shows RT-PCR analysis for SMN-full length and SMNΔ7 on treated mice and controls. As shown in FIG. 12(a,b), the level of exon 7-retaining full length SMN increased significantly in the mice treated with the inventive Morpholino modified E1 ASOs.

Example 3.

[0051] A single intracerebroventricular (ICV) injection of E1^{MO}-ASO (Element 1 v1.00; SEQ ID NO: 6) in the relatively severe mouse model of SMA (SMNΔ7 mouse model) elicited a robust induction of SMN protein and mean life span was extended ~300% following a single dose, consistent with large weight gains and a correction of the neuronal pathology. Additionally, E1^{MO}-ASO (Element 1 v1.00; SEQ ID NO: 6) treatment in an intermediate SMA mouse (SMN^{RT} mouse model) significantly extended life span by nearly 700% and weight gain was comparable to the unaffected animals. While a number of experimental therapeutics have targeted the ISS-N1 element of SMN2 pre-mRNA, the development of E1 ASOs provides a new molecular target for SMA therapeutics that dramatically extends survival in two important pre-clinical models of disease.

MATERIALS AND METHODS

Animal procedures and ASO delivery

[0052] Animals were housed and treated in accordance with Animal Care and Use Committee guidelines of the University of Missouri, Columbia, MO, USA. The colony was maintained as heterozygote breeding pairs under specific pathogen free conditions. SMNΔ7 (*SMNΔ7*$^{+/+}$;*SMN2*$^{+/+}$;*Smn*$^{-/-}$) and SMN^{RT} (*SMN^{RT+}*;*SMN2*$^{+/+}$;*Smn*$^{-/-}$) mice were genotyped on the day of birth (P1) using standard PCR protocol (JAX® Mice Resources) on tail tissue material. The following primer sets were used: for the mouse *Smn* gene, mSmn-WT forward (5'-tctgtgttcgtgcgtggtgacttt-3') (SEQ ID NO: 19) and mSmn-WT reverse (5'-cccaccacctaagaaagcctcaat-3') (SEQ ID NO: 20) and for the *Smn* knockout SMN1-KO forward (5'-ccaacttaatcgccttgcagcaca-3') (SEQ ID NO: 21) and SMN1-KO reverse (5'-aagcgagtggcaacatggaaatcg-3') (SEQ ID NO: 22). ICV injections were performed on P2, as previously described (Coady, T.H., et al., 2008; Osman, E.Y., et al., 2012; Passini, M.A., et al., 2011).

[0053] Mice were immobilized via cryoanesthesia and injected using μL calibrated sterilized glass micropipettes. The injection site was approximately 0.25 mm lateral to the sagittal suture and 0.50-0.75 mm rostral to the neonatal coronary suture. The needles were inserted perpendicular to the skull surface using a fiber-optic light (Boyce Scientific Inc.) to

aid in illuminating pertinent anatomical structures. Needles were removed after 10 seconds of discontinuation of plunger movement to prevent backflow. Treated animals were placed in a warmed container for recovery (5-10 minutes) until movement was restored. Single injections of 2 μL of the Morpholino modified E1^{MO}-ASOs were delivered via intracerebroventricular injections (ICV) as described above for all mice. Time-to-right (TTR) reflex tests were conducted as previously described (Butchbach, M.E., et al., 2007). Each pup was placed onto its back and the time it takes to right itself on the ground was recorded. The test was terminated at 30 seconds and if an animal had not turned by this time, it was recorded as 'Failure'. Righting reflex measurement were recorded daily starting at P7 since unaffected animals start to turn over at this time. For grip strength assessment, a grasping response test was utilized. Each pup's front paws were placed on a wire mesh (1 cm^2 grids) and gently dragged horizontally along the mesh (BioSeb Model BP32025, Vitrolles, FR, EU & Pinellas Park, FL, USA). Any resistance felt was scored as a positive response. The strength of the animal holding onto the mesh before release was recorded in grams. Grip strength was measured every 3-4 days starting on P25. Motor activity and coordination were measured by utilizing rotarod treadmill for mice (IITC Rotarod Series 8, IITC Life Science Inc., CA, USA). The animals were placed on textured drums to avoid slipping. When the tested animal dropped onto the individual sensing platform below, the test results were recorded in seconds. Measurements were performed every 3-4 days starting on P25.

Element 1 antisense oligonucleotides v1.00 (E1^{MO}-ASO v1.00)

[0054] The following oligos were modified at every base with Morpholino chemistry groups (GeneTools L.L.C., Philomath, OR 97370 USA); E1^{MO}-ASO (26-mer) 5'-CUA UAU AUA GAU AGU UAU UCA ACA AA -3' (SEQ ID NO: 23), and negative scrambled control provided and tested by GeneTools L.L.C. (25-mer), 5'- CCU CUU ACC UCA GUU ACA AUU UAU A-3' (SEQ ID NO: 24).

Immunohistochemistry of neuromuscular junctions (NMJs)

[0055] Immunochemistry and NMJ analysis were performed following a modified protocol described in detail previously (Cobb, M.S., et al., 2013; Ling, K.K., et al., 2012). Three animals from each treatment and control groups at age P12 were anaesthetized by anesthetic inhalant Isoflurane® USP, VetOne™ (1-chloro-2, 2,2-trifluoroethyl difluoromethyl ether; 50 mg/kg) followed by transcardiac perfusion with Phosphate Buffered Saline (PBS) solution (Dulbecco's, Gibco®, LifeTechnologies™ Carlsbad, CA, USA), and fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA). Whole-mount preparations were done by dissecting and examining the *longissimus capitis* muscle. Tissues were stained using specific antibodies including anti-neurofilament (1:2000; Chemicon®, EMD Millipore, Billerica, MA, USA) and anti-synaptophysin (1:200, LifeTechnologiesTM Carlsbad, CA, USA). Acetylcholine receptors (AChRs) were labeled with Alexa Fluor 594-conjugated α-bungarotoxin (LifeTechnologiesTM Carlsbad, CA, USA). Muscle preparations were viewed using a laser scanning confocal microscope (40x objective; 0.8NA; Zeiss LSM Model 510 META, Carl Zeiss, Jena, Germany, EU). From the confocal microscopy, Z-series stack images of immunostained whole-mount muscles were obtained at sequential focal planes 1 μm apart and merged using microscope integrated software and despeckled by ImageJ software, Fiji (Schindelin, J., et al., 2012).

RT-PCR assays

[0056] Total RNA was isolated from brain tissues harvested on P7 and homogenized using TRIzol reagent (LifeTechnologies™ Carlsbad, CA, USA). Two micrograms of total RNA was used to generate first-strand cDNA by using 100ng of random primers, 2 microliters dNTP (10mM) Mix; 4 microliters of 5x first-strand buffer, 1.0 microliter DTT (0.1 M) and 1.0 microliter SuperScript™ III Reverse Transcriptase (200U per microliter) (LifeTechnologies™ Carlsbad, CA, USA) at 50°C/50 min followed by reaction inactivation at 70°C/15 min. Cycling conditions were as follows: an initial denaturation step (94°C/3 min), 30 cycles (94°C/30 sec; 60°C/0.5 min; 72°C/1 min), and a final extension step (72°C/10 min). Reaction products were resolved by electrophoresis through a 2.0 % agarose gel (GeneMate, BioExpress, Kaysville, UT, USA) and visualized by ethidium bromide staining on FOTODYNE™ Imaging Systems (Hartland, WI, USA). For cDNA controls specific plasmids pCIExSkip and pCIFL were used (Lorson, C.L., et al., 1999).

Western blots

[0057] For the SMNΔ7 mouse Western blots, indicated tissues were collected at selected time points (P7) and immediately frozen in liquid nitrogen. Tissue samples were placed at -80°C until ready for analysis. Roughly 100 mg of tissue was homogenized in JLB buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM $NaH_2(PO_4)$, 25 mM NaF, 2 mM EDTA, 10% glycerol, 1% Triton X100, and protease inhibitors (Roche, Indianapolis, IN, USA)). Equal amounts of protein were separated on 12% SDS-PAGE gels. SMN immunoblots were performed using a mouse SMN specific monoclonal antibody

(BD Biosciences, San Jose, CA, USA) diluted 1:300 in TBST (Tris-buffered Saline Tween20 (10mM Tris-HCl, pH7.5, 150mM NaCl, 0.2% Tween20)) in 1.5% dry milk. Then blots were visualized by chemiluminescence on a Fujifilm imager LAS-3000 (FujiFilm^{USA}, Hanover Park, IL, USA) and the corresponding software. To verify equal loading the Westerns were then stripped using H_2O_2 for 30 minutes at room temperature and re-probed with anti-β-actin rabbit and anti-rabbit HRP secondary antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA). Western blots were performed in quadruplicate or more and representative blots are shown.

RESULTS

[0058] FIG. 13 illustrates targeting of the intronic repressor Element 1 with morpholino modified ASOs. FIG. 13 is a schematic representation of specific morpholino-modified ASO targeting the intronic repressor Element 1 (E1^{MO}-ASO). A specific design of an E1^{MO}-ASO is illustrated with the antisense domains consisting of two non-sequential target antisense sequences targeting the intronic Element 1 repressor. In addition, several previously published ASO sequences with different modified chemistries and targeting the intronic silencer ISS-N1 are also shown.

[0059] FIG. 14 shows an increase in full-length SMN transcript after E1^{MO}-ASO treatment. RT-PCR image showing full-length SMN in three individual animals. The plasmids pCIExSkip and pCIFL were used for cDNA controls.

[0060] FIG. 15 shows that injection of morpholino based ASO targeting the Element1 repressor increased total SMN protein in the Δ7SMA mouse model. Single ICV injection of E1^{MO}-ASO increase SMN protein levels. Western blots (n=5) for each treatment group were performed on brain, spinal cord and muscle tissues at P7.

[0061] FIG. 16a shows that severe SMNΔ7 SMA mice showed significant improvement in survival and longevity after injections with E1^{MO}-ASO oligos. Kaplan-Meier survival curves were constructed from the various treatment groups and the routes of delivery as indicated. Log-rank (Mantel-Cox) statistics were applied for comparisons between groups where $p<0.0001$ for all treatment groups compared to untreated animals, with the exception of E1^{MO}-ASO IP injected animals when compared to the untreated controls ($p=0.0526$). FIG. 16b shows survival curves demonstrating a significant increase in life expectancy for E1^{MO}-ASO ICV, ICV&ICV and ICV&IP injected animals with increases in median survival to 39, 54 and 54 days respectively. Some tail necrosis was displayed by the ICV injected SMA animals around day 40-45 (not shown).

[0062] FIG. 17 shows that E1^{MO}-ASO treatment results in a significant weight gain. Referring to FIG. 17, starting at P7, E1^{MO}-ASO injected animals were heavier than untreated, scrambled and IP only injected SMA controls. Total body weight was measured daily for all animal groups post injection.

[0063] FIG. 18a shows the percent weight gained from birth to peak was also compared between groups treated. FIG. 18b shows statistical significance between each treatment group. Statistical significance in percent weight gain from birth to peak after E1^{MO}-ASO treatment. P-values are shown for each treatment group. Student's t-Test was used to compare each group against all treatment and control animals. P values were rounded to the sixth decimal point.

[0064] FIG. 19 shows individual weights on P12 for all controls and animals treated with E1^{MO}-ASO.

[0065] FIG. 20 is a bar graph showing the percent of all tested animal groups able to right themselves from a prone position. By P14, more than fifty percent of all ICV, ICV&ICV, and ICV&IP injected were able to right themselves.

[0066] FIG. 21a is a graph representing raw data of the average time to right from P7 to P25. Animals injected with E1^{MO}-ASO ICV, E1^{MO}-ASO ICV&ICV and E1^{MO}-ASO ICV&IP were able to right themselves within 20 seconds after 2 weeks. FIG. 21b is a scatter plot of TTR performance of mice injected with E1^{MO}-ASO. To highlight the performance of individual mice, TTR values are shown for P12.

[0067] FIG. 22 shows grip strength measurements in grams. Treated animals were compared to their unaffected littermates. Measurements were taken from P25 through P77.

[0068] FIG. 23 shows results of the rotarod performance test. The test was used to measure riding time parameter (in seconds) of the E1^{MO}-ASO treated animals and compared with the times of their age-matched unaffected controls. Measurements were taken from P25 through P77.

[0069] Improvement in neuromuscular junctions (NMJs) pathology. The *longissimus capitis* (LC) muscles from ICV injected and control animals at P12 were immunostained for nerve terminals with anti-neurofilament/anti-synaptophysin [Nerve/Syn] and motor endplates with α-bungarotoxin. While the untreated SMNΔ7 mice displayed typical severe denervation, E1^{MO}-ASO treatment substantially restored NMJ's pretzel-like structures (not shown).

[0070] SMN protein induction in the milder mouse model of SMA (SMN^{RT}). FIG. 24a is a Western blot (n=3) showing increased SMN in spinal cord tissue of five (5) ICV injected animals with E1^{MO}-ASO. FIG. 24b shows Western blot quantification. Bar graph showing no significant difference in protein induction between unaffected and treated milder type SMA^{RT} mice.

[0071] SMN^{RT} mice injected with E1^{MO}-ASO, showed significant improvement in survival and weight gain. FIG. 25 shows that the treated SMN^{RT} animals (labeled "SMN^{RT} E1^{MO}-ASO") and the unaffected littermates (labeled "SMN^{RT} Unaffected") were substantially more vigorous and lived more than 180 days compared to the untreated mice (labeled "SMA^{RT} Untreated"). The Kaplan-Meier survival curve depicts an identical in life expectancy for unaffected and treated

SMN^{RT} mice. Animals were culled after 180 days.

[0072] FIG. 26 shows a significant increase in the average weight of SMN^{RT} animal model after treatment with E1^{MO}-ASO.

[0073] FIG. 27 show a comparison of the percent weight gained from birth to peak between animal groups. For statistical significance between each treatment group, Student's T-Test was used and p-values are shown for E1^{MO}-ASO SMN^{RT} animals.

[0074] FIG. 28 shows individual weights on P12. Weights of treated mice are comparable to the weights of unaffected age-matched littermates.

[0075] TTR, muscle strength, balance, and motor-planning measurements show significant improvement in E1^{MO}-ASO treated SMN^{RT} mice. FIG. 29 shows the percent of the tested animals able to right themselves compared to untreated mice where the TTR reflex is delayed. By P9, all treated and unaffected animals were able to right themselves.

[0076] FIG. 30 shows the average time to right from P7 to P25 for all experimental groups. SMN^{RT} mice injected with E1^{MO}-ASO were able to right themselves within 10 seconds after 10 days.

[0077] FIG. 31 is a scatter plot of TTR performance of SMN^{RT} mice injected with E1^{MO}-ASO. Time-to-right performance of individual mice at age P12 shows that treated animals can successfully turn themselves within 5 seconds.

[0078] FIG. 32 shows grip strength measurements in grams. Treated animals were compared to their unaffected littermates. Measurements were taken from P25 through P108.

[0079] FIG. 33 shows results of the rotarod performance test. The test was used to measure riding time parameter (in seconds) of the E1^{MO}-ASO treated animals and compared with the times of their age-matched unaffected controls. Measurements were taken from P26 through P110.

[0080] FIG. 34 is RT-PCR quantification showing the percent increase in full length SMN transcript in three SMA animals after treatment with E1^{MO}-ASO.

[0081] FIG. 35 is Western blot quantification showing the percent increase in SMN protein induction was compared to the unaffected and untreated control group and plotted in a bar graph. (n=5).

[0082] Delayed tail necrosis in E1^{MO}-ASO ICV/IP injected animals. After delivery via the combinatorial routes of ICV and IP injections, the treated animals exhibited a delayed tail necrosis (day 60-65) (not shown). E1^{MO}-ASO ICV injected SMN^{RT} animals were indistinguishable from their unaffected littermates in movement and behavior pattern. Necropsy of male SMN^{RT} mouse treated with E1^{MO}-ASO at age 175 days showed significant internal organ abnormalities such as deformed heart, subcutaneous fluid retention, and smaller kidneys. Enlarged and swollen bladder were clearly evident (not shown).

Element 1 as an ASO target

[0083] To expand the repertoire of potential targets for SMA therapeutics, Morpholino-modified ASOs (E1^{MO}-ASOS) were developed that target the E1 repressor, a region distinct from the ISS-N1 repressor which has been the focus of the overwhelming majority of ASO strategies (FIG. 13).

[0084] Following a single injection into the central nervous system via intracerebroventricular (ICV) delivery of the E1^{MO}-ASO, pre-mRNA exon-skipping from the SMN2 transgene was significantly reduced in total RNA isolated from brain tissue, resulting in a nearly 3-4 fold increase in full-length SMN transcript in each of three animals examined.

[0085] FIG. 14 shows the increase in full-length SMN transcript after E1^{MO}-ASO treatment. RT-PCR image showing full-length SMN in three individual animals. The plasmids, pCIExSkip and pCIFL, were used for cDNA controls.

[0086] The mice used in this experiment were phenotypically wild type (unaffected), but carried the human genomic SMN2 gene. To determine if SMN levels increased similarly, a single ICV injection was delivered to SMNΔ7 (*SMNΔ7*$^{+/+}$;*SMN2*$^{+/+}$;*Smn*$^{-/-}$) pups on P2 and protein extracts were collected from brain, spinal cord, and skeletal muscle (Musculus gastrocnemius). The SMNΔ7 model, which has an average life span of 12-14 days, has been extensively characterized and utilized for a number of translational studies (Le, T.T., et al., 2005; Osborne, M. and Lutz, C., 2013; Lorson, M.A. and Lorson, C.L., 2012). In each treated animal examined, SMN protein levels were elevated several fold compared to untreated SMA animals, although wildtype tissue still contained slightly higher levels of SMN.

Phenotypic correction in severe SMA mice

[0087] To determine whether delivery of the E1^{MO}-ASO to SMA mice on P2 improved the phenotype; survival, weight gain, righting reflexes and strength measurements were collected. A relatively low dose (2 mM) of ASO was selected, comparable to the "low" dose from a previously published report examining ISS-N1 ASOs (Porensky, P.N., et al., 2012). Untreated SMA mice lived less than 2 weeks, similar to a cohort treated with a control ASO consisting of the scrambled sequence. Similarly, delivery of the E1^{MO}-ASO via a single intraperitoneal (IP) injection failed to extend survival beyond 1-2 days. However, a single ICV injection of E1^{MO}-ASO led to nearly a 400% improvement in life span, with more than one third of the treated animals living beyond 50 days. When we looked at the life span of the negative control group

animals, we observed no difference between our untreated and the scrambled Morpholino injected animals. SMA animal models exhibit extensive peripheral defects, particularly in severe models. To determine whether E1^{MO}-ASO treatment would rescue peripheral defects, a combinatorial treatment of ICV and IP injections was performed: two injections of the 2 mM ASO were delivered via ICV and an IP injection. In a separate cohort, two ICV injections were administered separated by 12 hours. The double-dosing resulted in a similar extension in survival, out to an average of 54 days, with more than one quarter of the animals living beyond 70 days. While the treated mice were highly ambulatory, distal necrosis, particularly of the tail, was observed in nearly all of the longer lived animals. In the ICV treated cohort, necrosis initiated at approximately P40-45, while ICV/IP treatment delayed necrosis onset to approximately P60. Collectively, these results demonstrate that the E1^{MO}-ASO can significantly extend survival in a severe mouse model of SMA.

[0088] E1^{MO}-ASO treatment resulted in significant weight gain compared to either untreated, scramble or IP-only ASO treated cohorts. The ICV/IP treated animals gained the most weight compared to the ICV or ICV/ICV groups, resulting in animals that achieved 15-18 grams. This was in stark contrast to the untreated, scramble or IP-only ASO treated cohorts that failed to thrive and were unable to achieve 5 grams. An additional measure of phenotypic correction used in the SMA field is the timed-righting response. Animals are placed on their backs and the time it takes to stand on four legs is recorded, as well as failed attempts. In the ICV, ICV/ICV, and ICV/IP cohorts, SMA treated animals improved significantly based upon the percentage of animals that could successfully turn over as well as the speed at which the animals successfully righted themselves.

[0089] Typically, SMA animals do not live long enough to perform gross motor function tests such as rotarod and grip strength; however, E1^{MO}-ASO treated mice lived long enough and were healthy enough to perform these assays. Following a one week period to acclimate to the equipment, grip strength and rotarod performance was collected for 16 consecutive days. Grip strength analysis revealed that the SMA E1^{MO}-ASO treated animals performed consistently, albeit with less force, compared to wild type animals. Rotarod performance also demonstrated that the ASO-rescued animals were not fully corrected compared to wild type animals especially at later time points. While the treated animals were never fully corrected compared to wild type animals, it is important to stress that their untreated (or scramble-treated cohorts) were dead weeks prior to these studies. This increasing discrepancy could in part be due to the development of tail necrosis as flexibility and/or loss of the tail would impact balance and rotarod performance.

[0090] An important hallmark of the SMA phenotype that directly relates to disease pathogenesis is the integrity of the neuromuscular junctions (NMJs). As expected, NMJs from untreated SMA mice appear immature, poorly developed and there was little overlap between the pre- and post-synaptic endplate. In contrast, the wild type and E1^{MO}-ASO-treated tissues exhibit well developed NMJs with a high degree of connectivity between the axons and the post-synaptic endplate. These results are consistent with the significant correction of the SMA phenotype at the organismal level and provide evidence that a molecular correction of SMN2 splicing using an E1^{MO}-ASO can profoundly reverse the severe SMA phenotype observed in SMNΔ7 mice.

Phenotypic correction in intermediate SMA mice

[0091] Testing therapeutics in more than one model of disease validates the molecular engagement of a specific target, demonstrates applicability to a broader range of the patient population, and sheds light upon the biology of the disease. To address these important parameters, a newly developed intermediate model of disease was examined. This model, SMN^{RT}, expresses low levels of SMN, lives approximately 32 days, and exhibits an intermediate phenotype in most cellular and organismal parameters of disease (Cobb, M.S., et al., 2013). Following a single ICV injection of the E1^{MO}-ASO (2 mM), SMN protein was increased significantly, approximately 8-10 fold above untreated levels in spinal cord extracts. To verify whether ICV delivery of E1^{MO}-ASO also extended survival of the milder form SMA animals, lifespan for the treatment groups were analyzed by Kaplan-Meier survival curve and compared to the lifespan of the animals from the control groups. ICV injections of the E1^{MO}-ASO significantly increased the average lifespan of the SMN^{RT} mice, compared to their aged-matched untreated control animals. In fact, all E1^{MO}-ASO treated animals were still alive at P175, at which point the experiment was stopped and animals were euthanized. The treated SMN^{RT} mice were phenotypically indistinguishable from their unaffected age-matched littermates within the first two months of their life span. Consistent with an early and robust increase of SMN, treated SMN^{RT} mice gained weight to near wild type levels during the first 4-5 weeks, and treated animals weighed as much as wild type animals beyond approximately 40 days. At nearly all time points, SMN^{RT} treated mice were able to right themselves more rapidly than untreated mice and were as efficient as the wild type animals at time points beyond P10. Differences in grip strength and rotarod performance were detected over the trial period and as the animals aged, a greater disparity was observed between SMN^{RT} treated mice and unaffected animals. Similar to the SMNΔ7 experiments, the initiation of tail necrosis later in life (at approximately P70-80 for the SMN^{RT} mice) may have negatively impacted their ability to perform in these assays.

[0092] The dose that was initially administered was a 2 μM ICV injection. However, a doubling of this dose via two ICV injections or an ICV+IP dosing further enhanced survival. Interestingly, the ICV+IP dosing was the most efficacious presumably because the IP administration allowed for a greater distribution to peripheral tissues. It is also important to

stress that the SMA mouse models do not necessarily reflect the frequency of peripheral complications in most SMA patients. Currently, all of the SMA models exhibit profound peripheral organ defects, while peripheral organ damage in SMA patients is largely restricted to very severe SMA cases.

[0093] While the invention has been described in connection with specific embodiments thereof, it will be understood that the inventive device is capable of further modifications. This patent application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth.

[0094] The invention comprises the following embodiments:

Embodiment 1. A composition for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA, the composition comprising an antisense oligonucleotide that comprises a sequence annealing to a first region and a second region of the SMN2 pre-mRNA, wherein the first region of the SMN2 pre-mRNA consists of the nucleotides between -134 to -90 relative to exon 7 of the SMN2 pre-mRNA and the second region of the SMN2 pre-mRNA consists of the nucleotides between -105 to - 45 relative to exon 7 of the SMN2 pre-mRNA.

Embodiment 2. The composition of Embodiment 1 wherein said antisense oligonucleotide comprises a Morpholino backbone.

Embodiment 3. The composition of Embodiment 1 wherein the antisense oligonucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 17 (v1.11), SEQ ID NO: 16 (v1.10), SEQ ID NO: 6 (v1.00), SEQ ID NO: 7 (v1.01), SEQ ID NO: 8 (v1.02), SEQ ID NO: 9 (v1.03), SEQ ID NO: 10 (v1.04), SEQ ID NO: 11 (v1.05), SEQ ID NO: 12(v1.06), SEQ ID NO: 13(v1.07), SEQ ID NO: 14 (v1.08). SEQ ID NO: 15 (v1.09), and SEQ ID NO: 18 (v1.12).

Embodiment 4. The composition of Embodiment 3 wherein said antisense oligonucleotide comprises a Morpholino backbone.

Embodiment 5. The composition of Embodiment 1 wherein the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and is complementary to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA, wherein the sequence of the antisense oligonucleotide that is complementary to the second region of the SMN2 pre-mRNA is 5' of the sequence of the antisense nucleotide that is complementary to the first region of the SMN2 pre-mRNA.

Embodiment 6. The composition of Embodiment 5 wherein said antisense oligonucleotide comprises a Morpholino backbone.

Embodiment 7. The composition of Embodiment 1 wherein the antisense oligonucleotide comprises:

i. a nucleotide sequence that is complementary to, except for having one or two nucleotide substitutions, at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and is complementary to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA;
ii. a nucleotide sequence that is complementary to at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and that is complementary, except for having one or two nucleotide substitutions, to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA; or
iii. a nucleotide sequence that is complementary to, except for having one or two nucleotide substitutions, at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and that is complementary, except for having one or two nucleotide substitutions, to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA.

Embodiment 8. The composition of Embodiment 7 wherein said antisense oligonucleotide comprises a Morpholino backbone.

Embodiment 9. The composition of Embodiment 5 wherein the entire sequence of the antisense oligonucleotide is not complementary to a wholly consecutive sequence of the SMN2 pre-mRNA sequence.

Embodiment 10. The composition of Embodiment 9 wherein said antisense oligonucleotide comprises a Morpholino backbone.

Embodiment 11. The composition of Embodiment 9 wherein the antisense oligonucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 17 (v1.11), SEQ ID NO: 16 (v1.10), SEQ ID NO: 6 (v1.00), SEQ ID NO: 7 (v1.01), SEQ ID NO: 11 (v1.05), SEQ ID NO: 12(v1.06), SEQ ID NO: 13(v1.07), SEQ ID NO: 14 (v1.08). SEQ ID NO: 15 (v1.09), and SEQ ID NO: 18 (v1.12).

Embodiment 12. The composition of Embodiment 11 wherein said antisense oligonucleotide comprises a Morpholino backbone.

Embodiment 13. The composition of Embodiment 7 wherein the entire sequence of the antisense oligonucleotide is not complementary to a wholly consecutive sequence of the SMN2 pre-mRNA sequence.

Embodiment 14. The composition of Embodiment 13 wherein said antisense oligonucleotide comprises a Morpholino backbone.

Embodiment 15. A method for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA, the method comprising the step of administrating to a subject the composition of any of Embodiments 1 to 14.

Embodiment 16. The method of Embodiment 15 wherein the composition is administered intracerebroventricularly, intraperitoneally, intravenously, or by a combinatorial administration thereof.

Embodiment 17. A method for treating Spinal Muscular Atrophy (SMA) in a human SMA patient, the method comprising the step of administrating to the patient an effective amount of a composition of any of Embodiments 1 to 14.

Embodiment 18. The method of Embodiment 17 wherein the composition is administered intracerebroventricularly, intraperitoneally, intravenously, or by a combinatorial administration thereof.

Embodiment 19. A composition for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA, the compositions comprising an antisense oligonucleotide with a sequence annealing to a first region and a second region of the SMN2 pre-mRNA, wherein the first region consists of the nucleotides between -134 to -120 relative to exon 7 of the SMN2 pre-mRNA and the second region consists of the nucleotides between -67 to -54 relative to exon 7 of the SMN2 pre-mRNA.

Embodiment 20. The composition of Embodiment 19 wherein said antisense oligonucleotide comprises a Morpholino backbone.

Embodiment 21. A method for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA, thereby modulating the splicing pattern of the SMN2 to generate full-length (exon-7-retaining) SMN comprising the step of administrating to a subject a composition comprising an antisense nucleotide with a sequence annealing to two distinct regions flanking E1 on the SMN2 pre-mRNA, whereas the regions consist of the nucleotides between -134 to -120 and -67 to -54 (relative to exon 7) and a Morpholino backbone.

SEQUENCE LISTING

<110> The Curators of the University of Missouri

<120> SMN2 Element 1 Antisense Compositions and Methods and Uses Thereof

<130> BET 19M0828

<150> US 61/853,820
<151> 2013-04-12

<160> 45

<170> PatentIn version 3.5

<210> 1
<211> 222
<212> DNA
<213> Homo sapiens

<400> 1
tgcaagaaaa ccttaactgc agcctaataa ttgttttctt tgggataact tttaaagtac 60

attaaaagac tatcaactta atttctgatc atattttgtt gaataaaata agtaaaatgt 120

cttgtgaaac aaaatgcttt ttaacatcca tataaagcta tctatatata gctatctatg 180

tctatatagc tatttttttt aacttccttt attttcctta ca 222

<210> 2
<211> 45
<212> DNA
<213> Homo sapiens

<400> 2
gtaaaatgtc ttgtgaaaca aaatgctttt taacatccat ataaa 45

<210> 3
<211> 87
<212> DNA
<213> Homo sapiens

<400> 3
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 4
<211> 87
<212> DNA
<213> Homo sapiens

<400> 4
tctatcgata tatatctatc gaaatatacc tacaattttt cgtaaaacaa agtgttctgt 60

aaaatgaata aaataagttg ttttata 87

<210> 5

<211> 39
<212> DNA
<213> Homo sapiens

<400> 5
ctatatatag atagttattc aacaaaacta gtaattttt 39

<210> 6
<211> 26
<212> DNA
<213> Homo sapiens

<400> 6
ctatatatag atagttattc aacaaa 26

<210> 7
<211> 25
<212> DNA
<213> Homo sapiens

<400> 7
tagatagctt tacattttac ttatt 25

<210> 8
<211> 25
<212> DNA
<213> Homo sapiens

<400> 8
tatggatgtt aaaaagcatt ttgtt 25

<210> 9
<211> 25
<212> DNA
<213> Homo sapiens

<400> 9
ctatatatag atagctttat atgga 25

<210> 10
<211> 25
<212> DNA
<213> Homo sapiens

<400> 10
cattttactt attttattca acaaa 25

<210> 11
<211> 25
<212> DNA
<213> Homo sapiens

<400> 11
gctttatatg gacattttac ttatt 25

<210> 12

<211> 25
<212> DNA
<213> Homo sapiens

<400> 12
gatgttaaaa agcgtttcac aagac 25

<210> 13
<211> 25
<212> DNA
<213> Homo sapiens

<400> 13
tatatggatg ttattattca acaaa 25

<210> 14
<211> 25
<212> DNA
<213> Homo sapiens

<400> 14
gcattttgtt tcacaagtta ttcaa 25

<210> 15
<211> 25
<212> DNA
<213> Homo sapiens

<400> 15
ctatatatag atagcgacat tttac 25

<210> 16
<211> 26
<212> DNA
<213> Homo sapiens

<400> 16
agatagcttt atatggattt attcaa 26

<210> 17
<211> 20
<212> DNA
<213> Homo sapiens

<400> 17
ctatatatag ttattcaaca 20

<210> 18
<211> 24
<212> DNA
<213> Homo sapiens

<400> 18
tttatatgga tgaagacatt ttac 24

<210> 19

<211> 24
<212> DNA
<213> Mus musculus

<400> 19
tctgtgttcg tgcgtggtga cttt 24

<210> 20
<211> 24
<212> DNA
<213> Mus musculus

<400> 20
cccaccacct aagaaagcct caat 24

<210> 21
<211> 24
<212> DNA
<213> Mus musculus

<400> 21
ccaacttaat cgccttgcag caca 24

<210> 22
<211> 24
<212> DNA
<213> Mus musculus

<400> 22
aagcgagtgg caacatggaa atcg 24

<210> 23
<211> 25
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 23
ccucuuaccu caguuacaau uuaua 25

<210> 24
<211> 26
<212> RNA
<213> Homo sapiens

<400> 24
cuauauauag auaguuauuc aacaaa 26

<210> 25
<211> 14
<212> RNA
<213> Homo sapiens

<400> 25
cuauauauag auag 14

<210> 26
<211> 12
<212> RNA
<213> Homo sapiens

<400> 26
uuauucaaca aa 12

<210> 27
<211> 15
<212> RNA
<213> Homo sapiens

<400> 27
cuuucauaau gcugg 15

<210> 28
<211> 18
<212> RNA
<213> Homo sapiens

<400> 28
ucacuuucau aaugcugg 18

<210> 29
<211> 20
<212> RNA
<213> Homo sapiens

<400> 29
auucacuuuc auaaugcugg 20

<210> 30
<211> 20
<212> RNA
<213> Homo sapiens

<400> 30
auucacuuuc auaaugcugg 20

<210> 31
<211> 21
<212> RNA
<213> Homo sapiens

<400> 31
gauucacuuu cauaaugcug g 21

<210> 32
<211> 25
<212> RNA
<213> Homo sapiens

<400> 32
guuagauuca cuuucauaau gcugg 25

<210> 33
<211> 87
<212> DNA
<213> Homo sapiens

<400> 33
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 34
<211> 87
<212> DNA
<213> Homo sapiens

<400> 34
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 35
<211> 87
<212> DNA
<213> Homo sapiens

<400> 35
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 36
<211> 87
<212> DNA
<213> Homo sapiens

<400> 36
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 37
<211> 87
<212> DNA
<213> Homo sapiens

<400> 37
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 38
<211> 87
<212> DNA
<213> Homo sapiens

<400> 38

atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 39
<211> 87
<212> DNA
<213> Homo sapiens

<400> 39
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 40
<211> 87
<212> DNA
<213> Homo sapiens

<400> 40
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 41
<211> 87
<212> DNA
<213> Homo sapiens

<400> 41
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 42
<211> 87
<212> DNA
<213> Homo sapiens

<400> 42
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 43
<211> 87
<212> DNA
<213> Homo sapiens

<400> 43
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 44
<211> 87
<212> DNA

<213> Homo sapiens

<400> 44
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

<210> 45
<211> 87
<212> DNA
<213> Homo sapiens

<400> 45
atattttgtt gaataaaata agtaaaatgt cttgtgaaac aaaatgcttt ttaacatcca 60

tataaagcta tctatatata gctatct 87

Claims

1. A composition for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA, the composition comprising an antisense oligonucleotide that comprises a sequence annealing to a first region and a second region of the SMN2 pre-mRNA, wherein the first region of the SMN2 pre-mRNA consists of the nucleotides between -134 to -90 relative to exon 7 of the SMN2 pre-mRNA and the second region of the SMN2 pre-mRNA consists of the nucleotides between -105 to - 45 relative to exon 7 of the SMN2 pre-mRNA, not including the composition comprising an antisense oligonucleotide that comprises the nucleotide sequence of SEQ ID NO: 17 or the nucleotide sequence of SEQ ID NO: 17 except for having one or two nucleotide substitutions.

2. The composition of Claim 1 wherein the antisense oligonucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 16 (v1.10), SEQ ID NO: 6 (v1.00), SEQ ID NO: 7 (v1.01), SEQ ID NO: 8 (v1.02), SEQ ID NO: 9 (v1.03), SEQ ID NO: 10 (v1.04), SEQ ID NO: 11 (v1.05), SEQ ID NO: 12(v1.06), SEQ ID NO: 13(v1.07), SEQ ID NO: 14 (v1.08). SEQ ID NO: 15 (v1.09), and SEQ ID NO: 18 (v1.12).

3. The composition of claim 1 wherein the antisense oligonucleotide comprises a nucleotide sequence that is complementary to at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and is complementary to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA, wherein the sequence of the antisense oligonucleotide that is complementary to the second region of the SMN2 pre-mRNA is 5' of the sequence of the antisense nucleotide that is complementary to the first region of the SMN2 pre-mRNA.

4. The composition of claim 1 wherein the antisense oligonucleotide comprises:

   i. a nucleotide sequence that is complementary to, except for having one or two nucleotide substitutions, at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and is complementary to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA;
   ii. a nucleotide sequence that is complementary to at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and that is complementary, except for having one or two nucleotide substitutions, to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA; or
   iii. a nucleotide sequence that is complementary to, except for having one or two nucleotide substitutions, at least eight consecutive nucleotides of the first region of the SMN2 pre-mRNA and that is complementary, except for having one or two nucleotide substitutions, to at least eight consecutive nucleotides of the second region of the SMN2 pre-mRNA.

5. The composition of claim 3 wherein the entire sequence of the antisense oligonucleotide is not complementary to a wholly consecutive sequence of the SMN2 pre-mRNA sequence.

6. The composition of claim 5 wherein the antisense oligonucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 16 (v1.10), SEQ ID NO: 6 (v1.00), SEQ ID NO: 7 (v1.01), SEQ ID NO: 11

(v1.05), SEQ ID NO: 12(v1.06), SEQ ID NO: 13(v1.07), SEQ ID NO: 14 (v1.08). SEQ ID NO: 15 (v1.09), and SEQ ID NO: 18 (v1.12).

7. The composition of claim 4 wherein the entire sequence of the antisense oligonucleotide is not complementary to a wholly consecutive sequence of the SMN2 pre-mRNA sequence.

8. The composition of any one of Claims 1 to 7 wherein said antisense oligonucleotide comprises a Morpholino backbone.

9. A composition of any of claims 1 to 8 for use for blocking the responsive activity of the Element 1 of the SMN2 pre-mRNA in a subject.

10. The composition for the use of claim 9 wherein the composition is administered intracerebroventricularly, intraperitoneally, intravenously, or by a combinatorial administration thereof.

11. An effective amount of a composition of any of claims 1 to 8 for use in treating Spinal Muscular Atrophy (SMA) in a human SMA patient.

12. The composition for the use of claim 11 wherein the composition is administered intracerebroventricularly, intraperitoneally, intravenously, or by a combinatorial administration thereof.

13. A composition for blocking the repressive activity of the Element 1 of the SMN2 pre-mRNA, the compositions comprising an antisense oligonucleotide with a sequence annealing to a first region and a second region of the SMN2 pre-mRNA, wherein the first region consists of the nucleotides between -134 to -120 relative to exon 7 of the SMN2 pre-mRNA and the second region consists of the nucleotides between -67 to -54 relative to exon 7 of the SMN2 pre-mRNA.

14. The composition of Claim 13 wherein said antisense oligonucleotide comprises a Morpholino backbone.

15. The composition of Claim 1 comprising an antisense nucleotide with a sequence annealing to two distinct regions flanking E1 on the SMN2 pre-mRNA, whereas the regions consist of the nucleotides between -134 to -120 and -67 to -54 (relative to exon 7) and a Morpholino backbone, for use for blocking the responsive activity of the Element 1 of the SMN2 pre-mRNA, thereby modulating the splicing pattern of the SMN2 to generate full-length (exon-7-retaining) SMN in a subject wherein the composition is administered intracerebroventricularly, intraperitoneally, intravenously, or by a combinatorial administration thereof and wherein said antisense oligonucleotide that comprises a Morpholino backbone is used for treating Spinal Muscular Atrophy (SMA) in a human SMA patient.

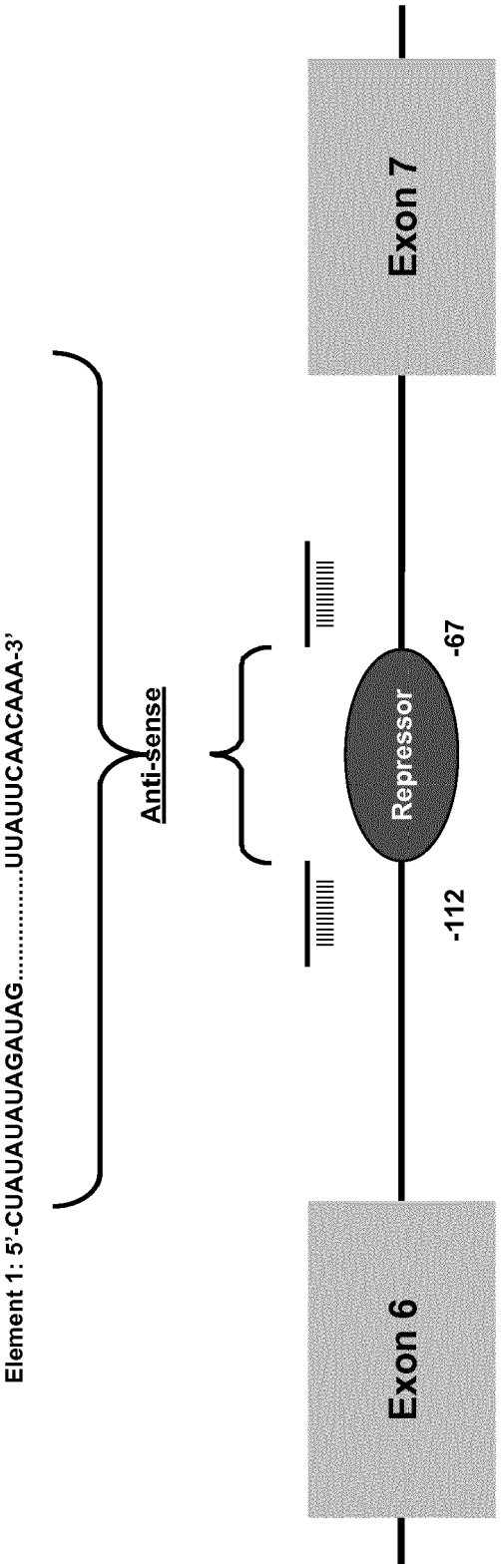

Element 1: 5'-CUAUAUAUAGAUAG.....UUAUUCAACAAA-3'
Anti-sense
Exon 7
Exon 6
Repressor
-67
-112
Element 1

FIG. 1


-46
-133
V1.00 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_33
V1.01 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_34
V1.02 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_35
V1.03 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_36
V1.04 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_37
V1.05 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_38
V1.06 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_39
V1.07 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_40
V1.08 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_41
V1.09 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_42
V1.10 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_43
V1.11 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_44
V1.12 3′-TCTATCGATATATATCTATCGAAATATACCTACAATTTTTCGTAAAACAAAGTGTTCTGTAAAATGAATAAAATAAGTTGTTTTATA-5′ Seq_45

FIG. 2

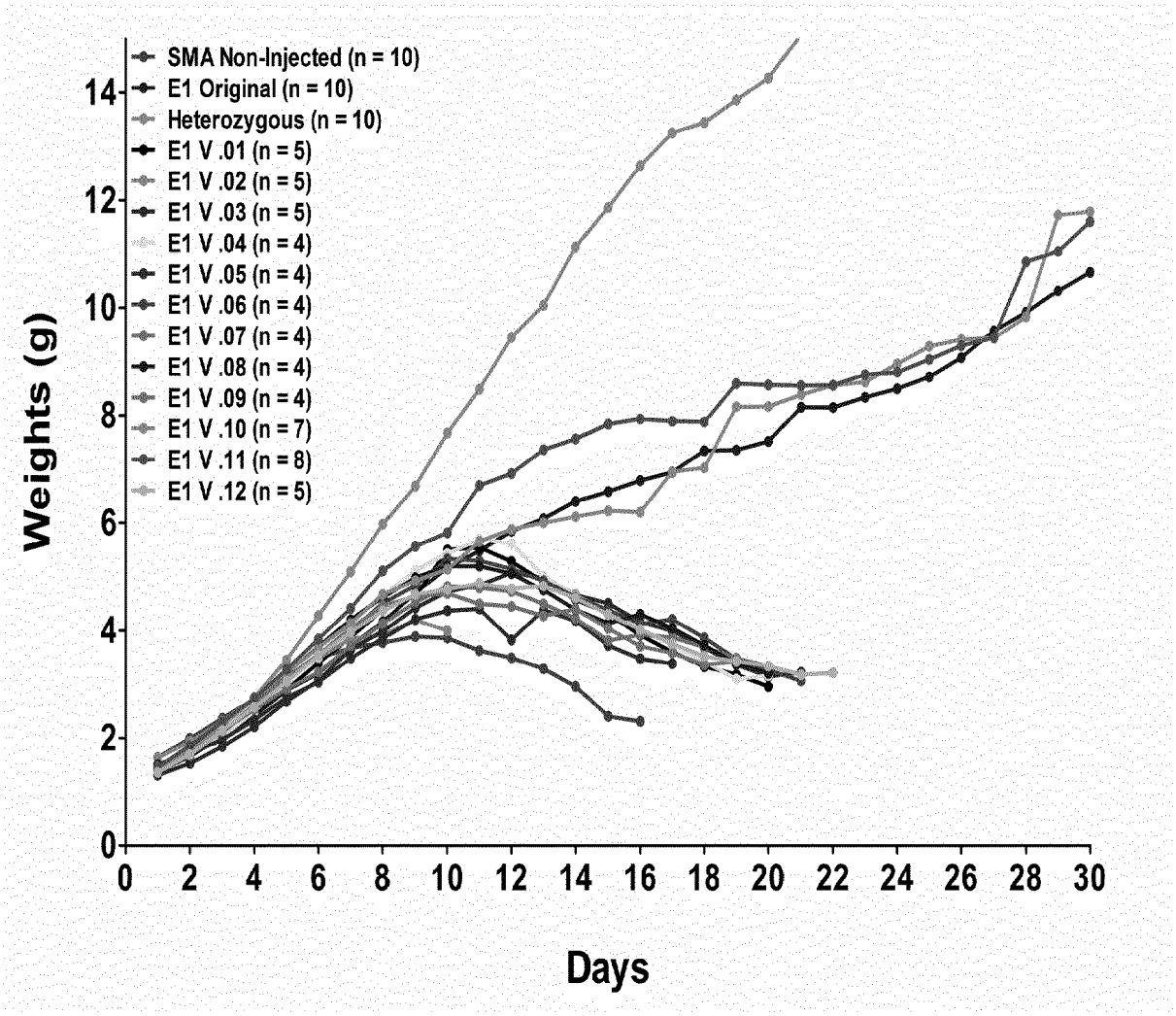

SMA Non-Injected (n = 10)
E1 Original (n = 10)
Heterozygous (n = 10)
E1 V .01 (n = 5)
E1 V .02 (n = 5)
E1 V .03 (n = 5)
E1 V .04 (n = 4)
E1 V .05 (n = 4)
E1 V .06 (n = 4)
E1 V .07 (n = 4)
E1 V .08 (n = 4)
E1 V .09 (n = 4)
E1 V .10 (n = 7)
E1 V .11 (n = 8)
E1 V .12 (n = 5)
Weights (g)
0
2
4
6
8
10
12
14
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days

FIG. 3

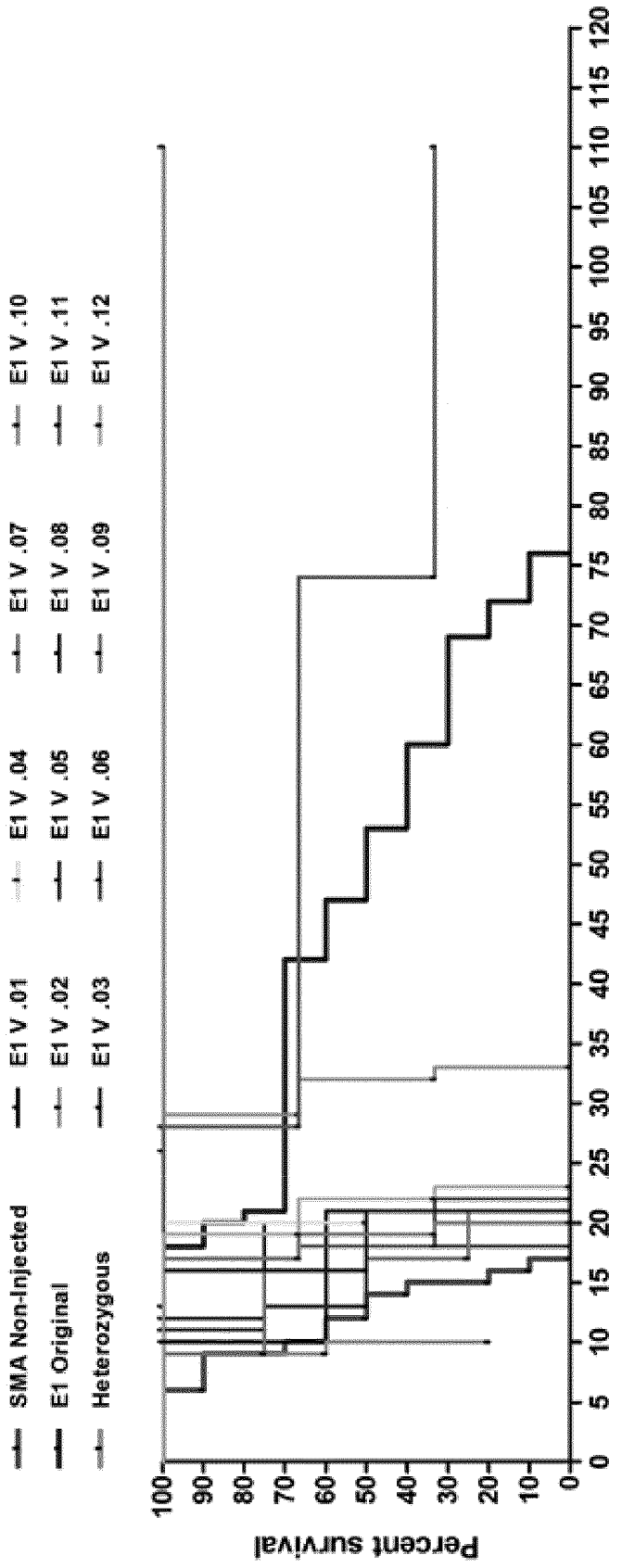

SMA Non-Injected
E1 Original
Heterozygous
E1 V .01
E1 V .02
E1 V .03
E1 V .04
E1 V .05
E1 V .06
E1 V .07
E1 V .08
E1 V .09
E1 V .10
E1 V .11
E1 V .12
Percent survival
Days
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120

FIG. 4

Percent Weight Gained from Birth to Peak of 2.0mM Concentration

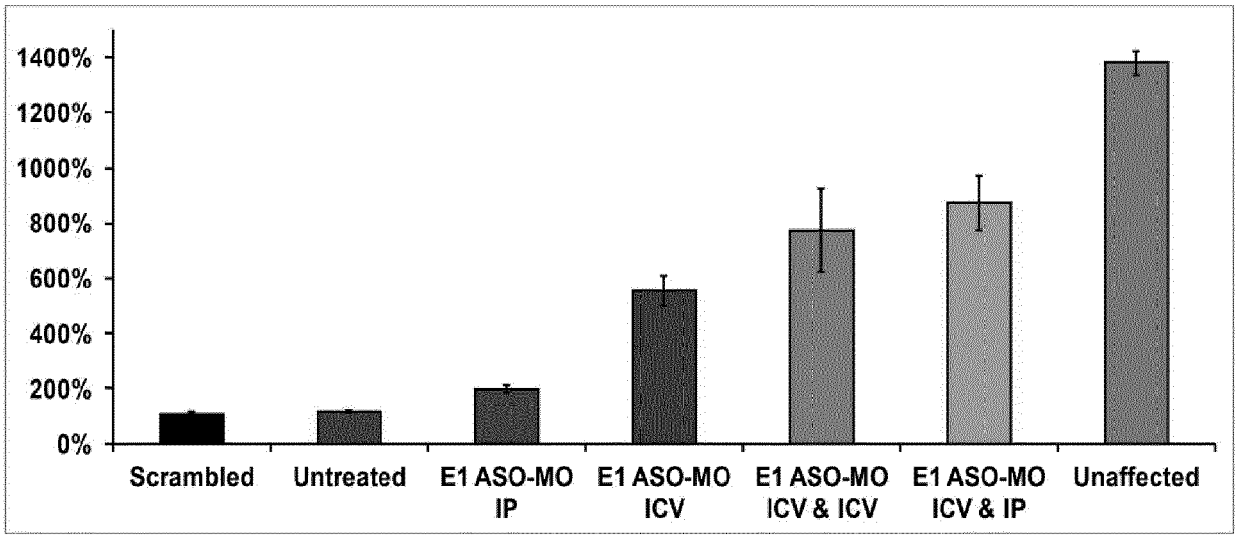


FIG. 5

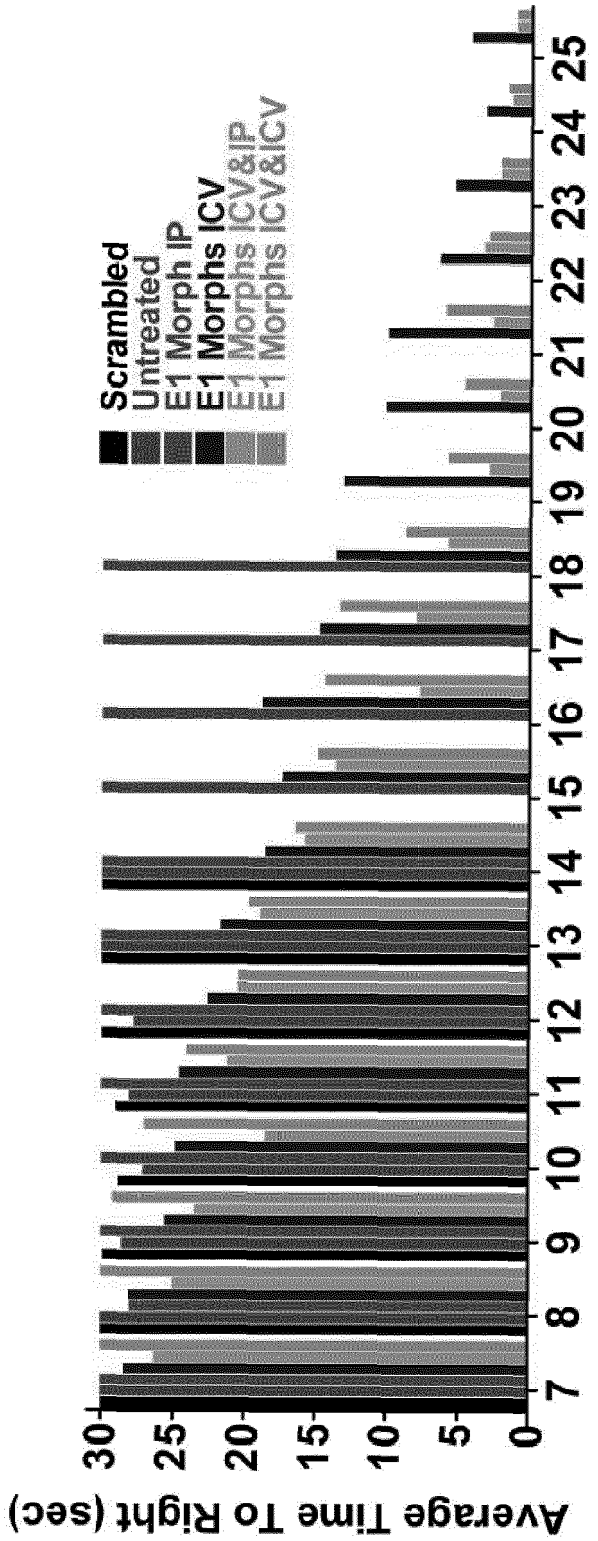

Scrambled
Untreated
E1 Morph IP
E1 Morphs ICV
E1 Morphs ICV&IP
E1 Morphs ICV&ICV
Average Time To Right (sec)
0
5
10
15
20
25
30
Days
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

FIG. 6

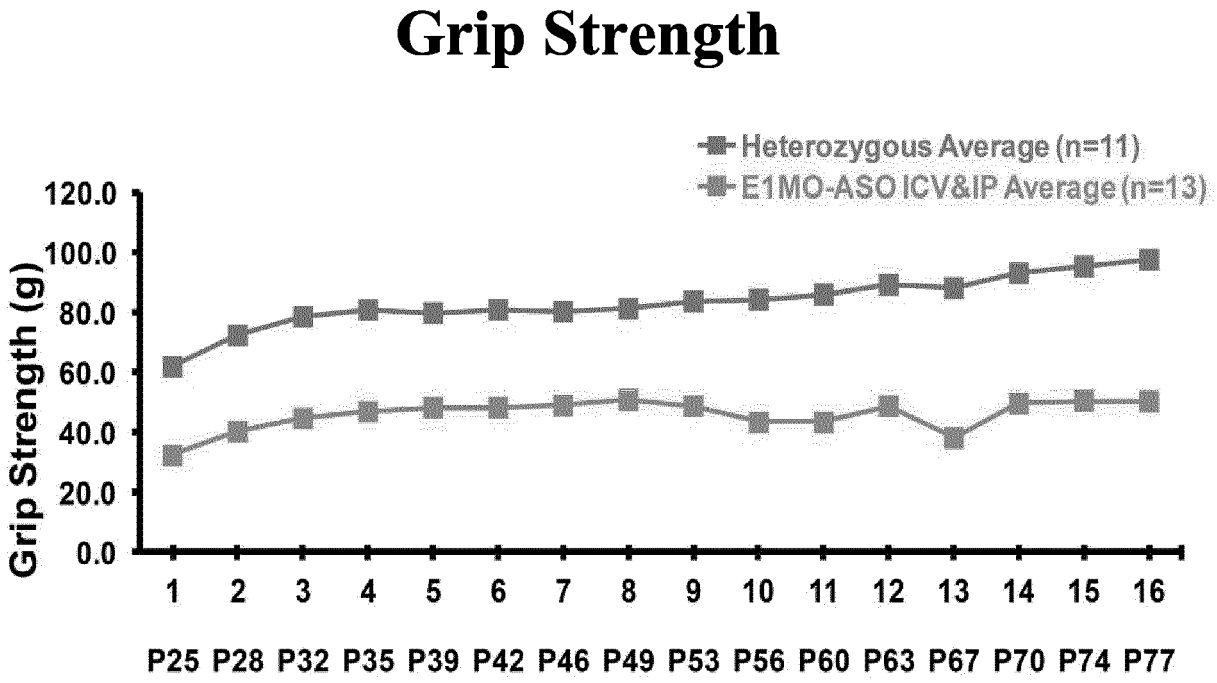

Grip Strength
Heterozygous Average (n=11)
E1MO-ASO ICV&IP Average (n=13)
Grip Strength (g)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
P25 P28 P32 P35 P39 P42 P46 P49 P53 P56 P60 P63 P67 P70 P74 P77

FIG. 7

Rotarod Performance Test

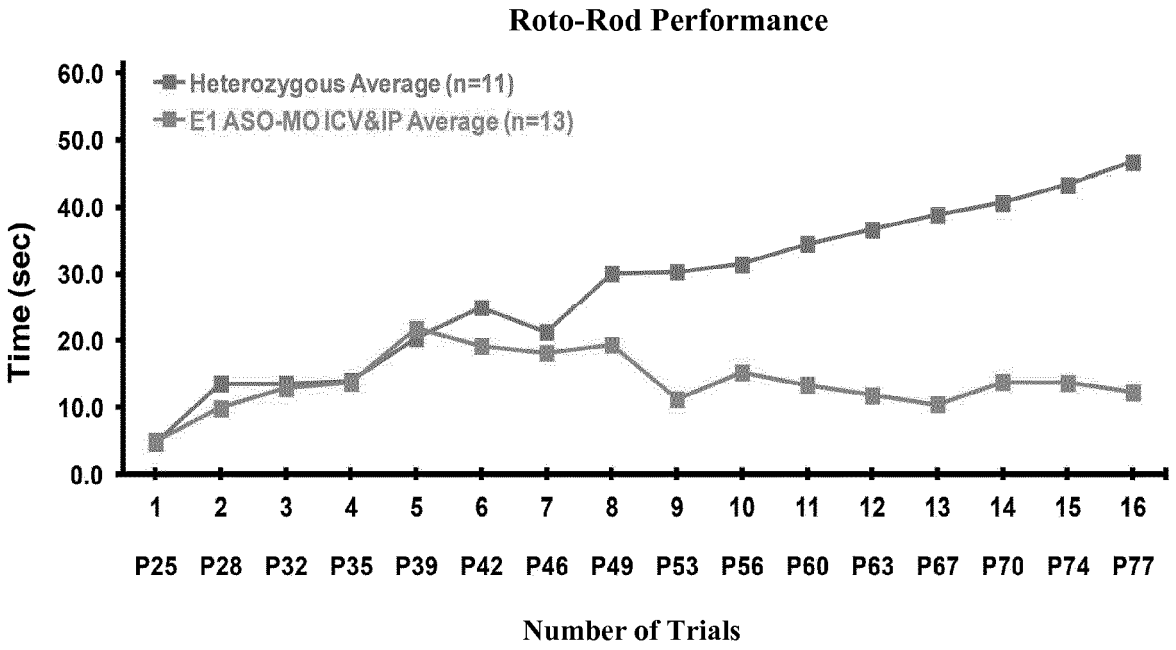
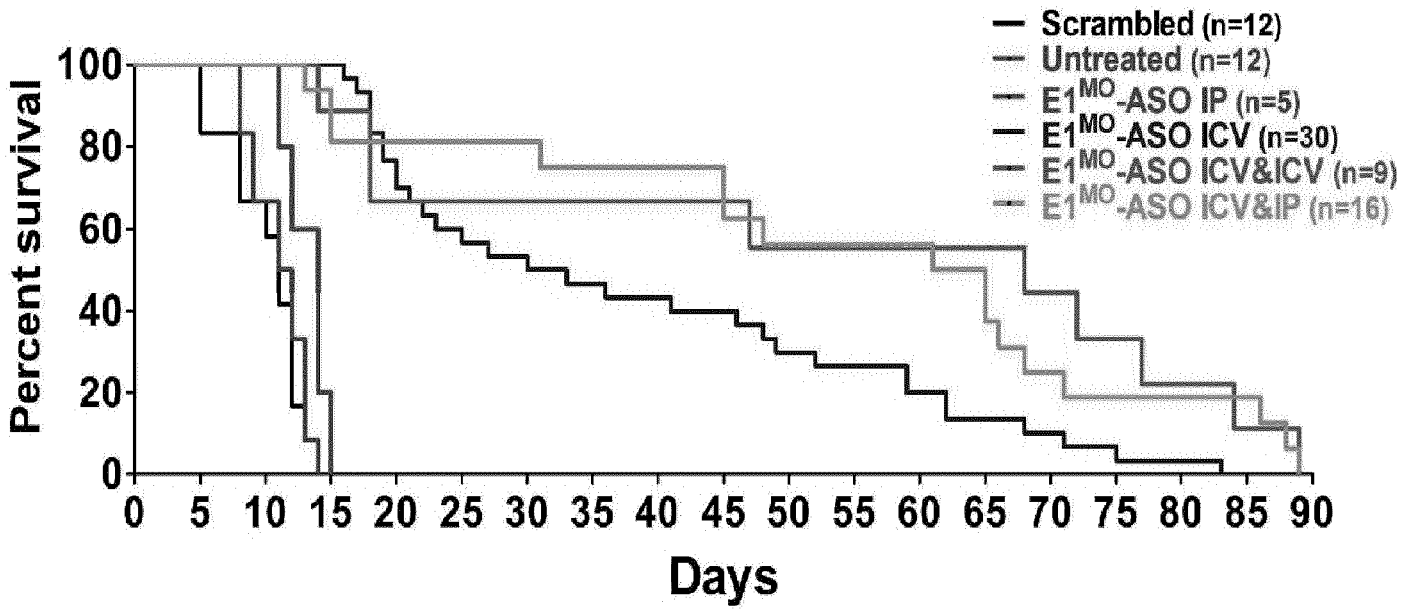


FIG. 8

Survival Data



Treatment	Scrambled	Untreated	EI ASO-MO IP	EI ASO-MO ICV	EI ASO-MO ICV & ICV	EI ASO-MO ICV & IP
Median Survival	10	11	13	39	54	54

FIG. 9

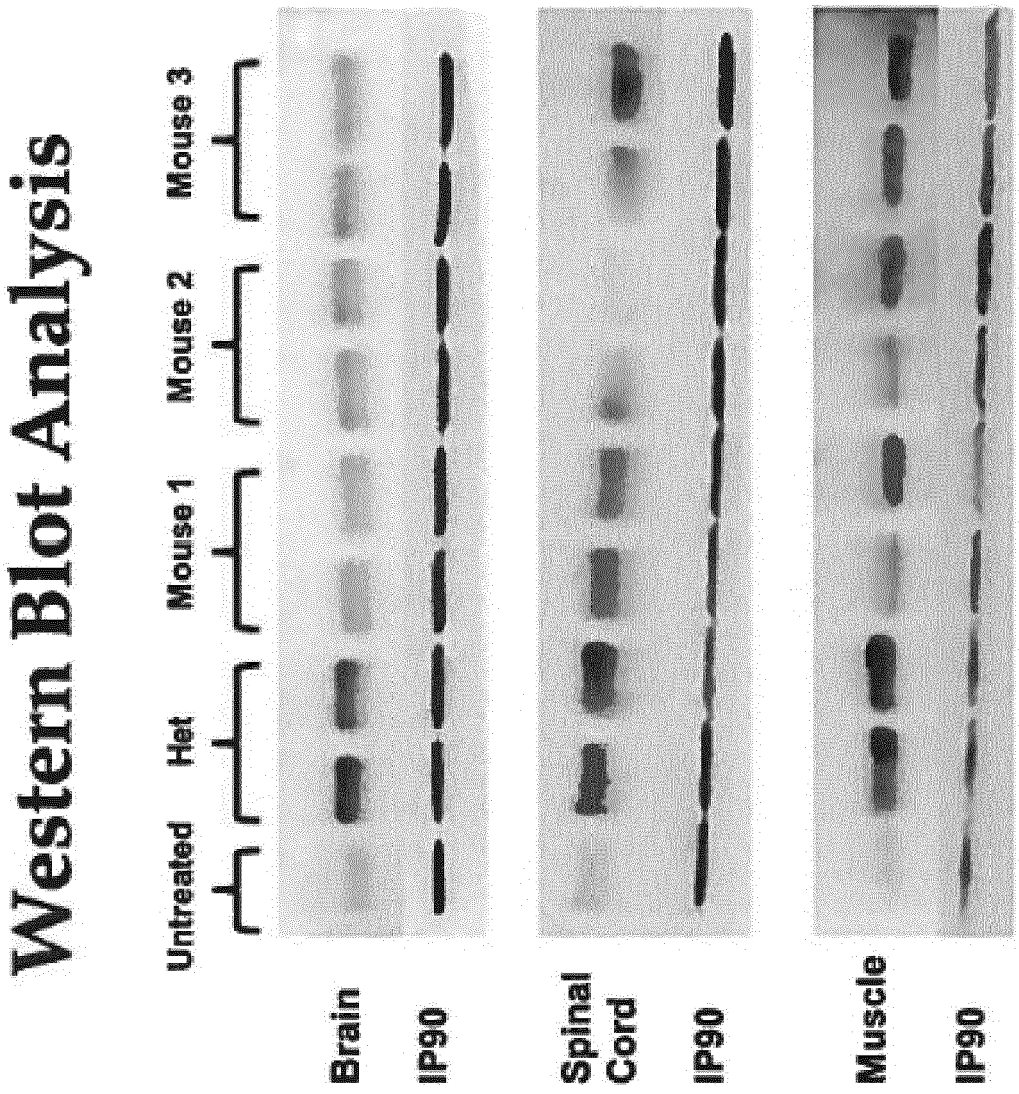

Western Blot Analysis
Untreated
Het
Mouse 1
Mouse 2
Mouse 3
Brain
IP90
Spinal Cord
IP90
Muscle
IP90

FIG. 10

Western Blot Analysis

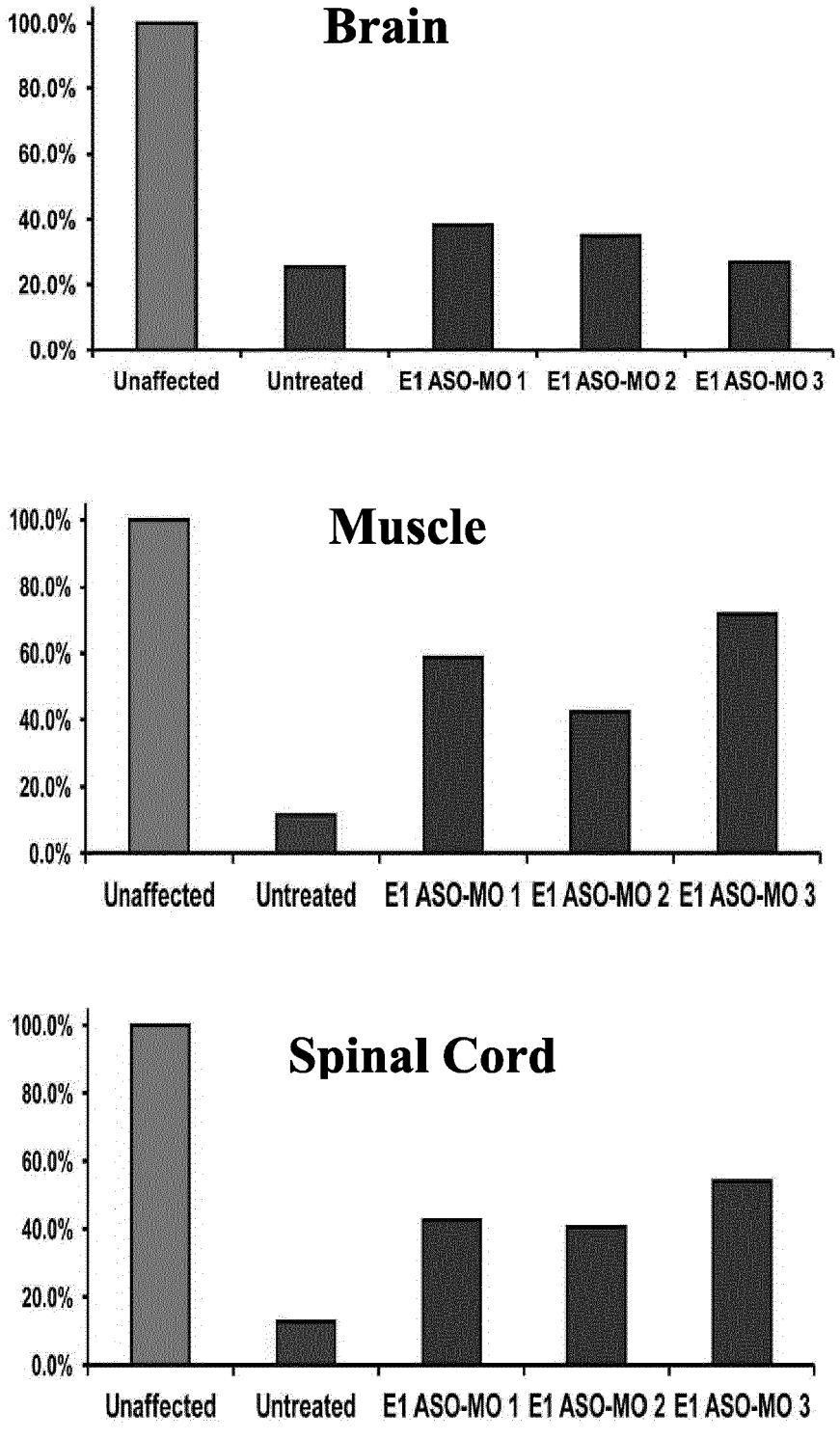


FIG. 11

RT-PCR for SMN-Full Length and SMN-Δ7

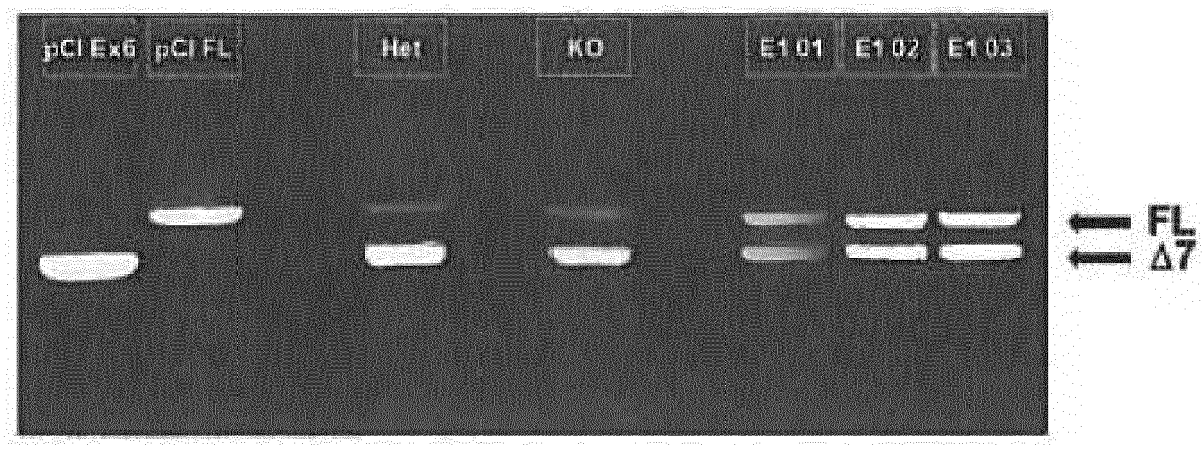


FIG. 12a

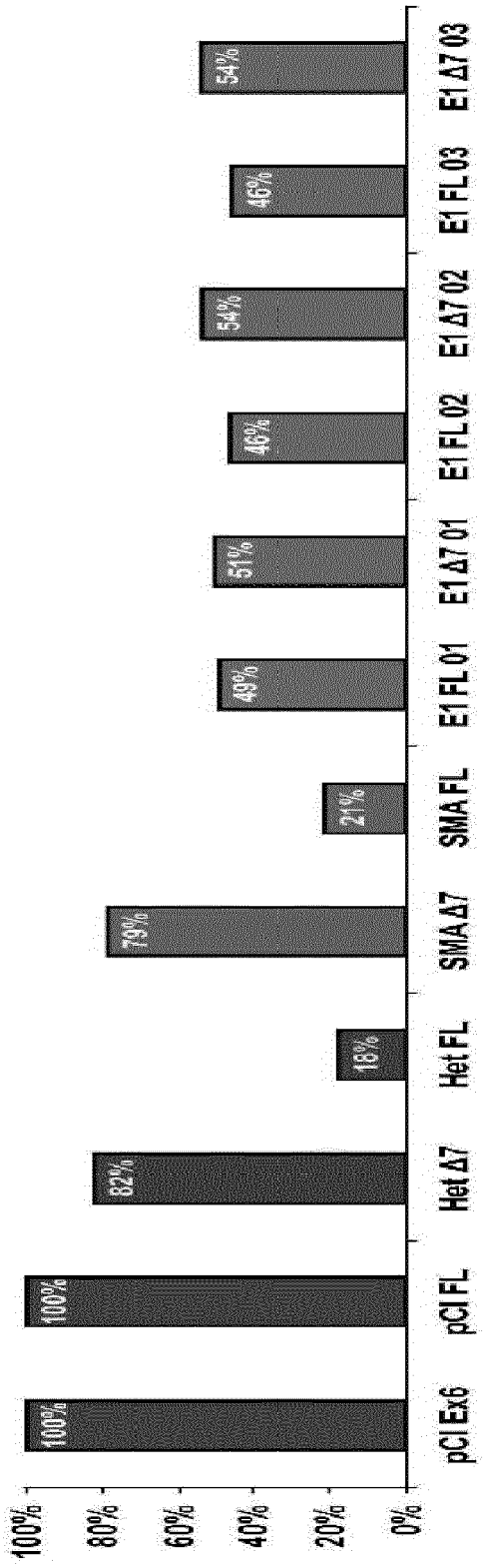

100%
80%
60%
40%
20%
0%
100%
100%
82%
79%
18%
21%
49%
51%
46%
54%
46%
54%
pCI Ex6
pCI FL
Het Δ7
Het FL
SMA Δ7
SMA FL
E1 FL 01
E1 Δ7 01
E1 FL 02
E1 Δ7 02
E1 FL 03
E1 Δ7 03

FIG. 12b

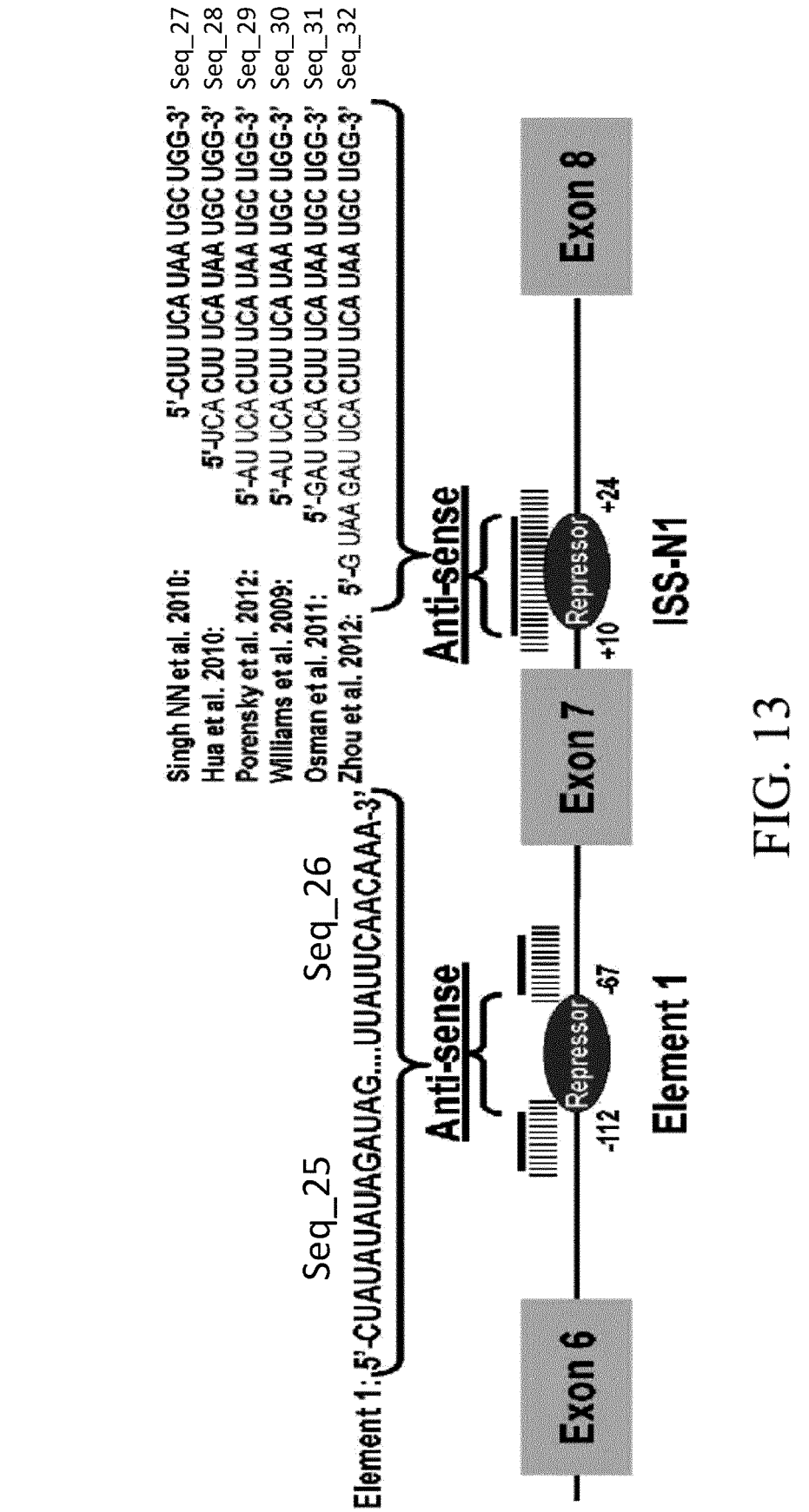

Singh NN et al. 2010: 5'-CUU UCA UAA UGC UGG-3' Seq_27
Hua et al. 2010: 5'-UCA CUU UCA UAA UGC UGG-3' Seq_28
Porensky et al. 2012: 5'-AU UCA CUU UCA UAA UGC UGG-3' Seq_29
Williams et al. 2009: 5'-AU UCA CUU UCA UAA UGC UGG-3' Seq_30
Osman et al. 2011: 5'-GAU UCA CUU UCA UAA UGC UGG-3' Seq_31
Zhou et al. 2012: 5'-G UAA GAU UCA CUU UCA UAA UGC UGG-3' Seq_32
Element 1: 5'-CUAUAUAUAGAUAG....UUAUUCAACAAA-3'
Seq_25
Seq_26
Anti-sense
Anti-sense
Repressor
Repressor
-112
-67
+10
+24
Exon 6
Exon 7
Exon 8
Element 1
ISS-N1

FIG. 13

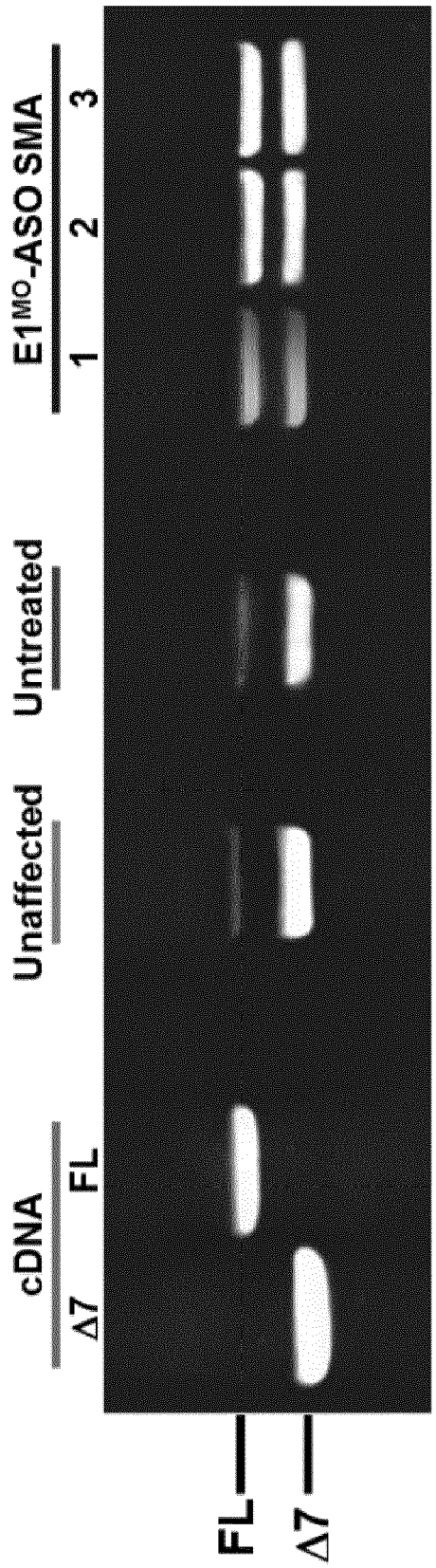


FIG. 14

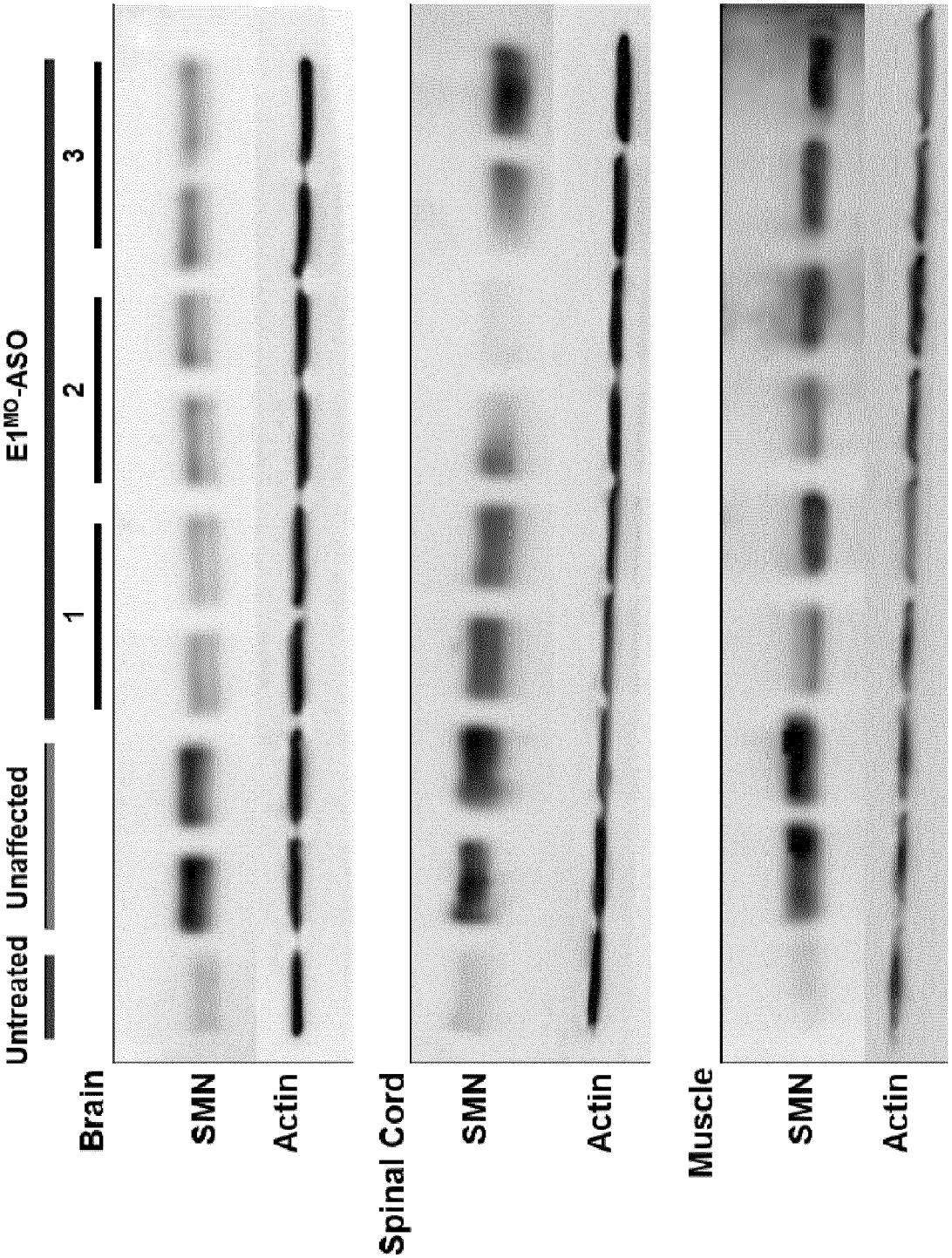

Untreated
Unaffected
E1MO-ASO
1
2
3
Brain
SMN
Actin
Spinal Cord
SMN
Actin
Muscle
SMN
Actin

FIG. 15

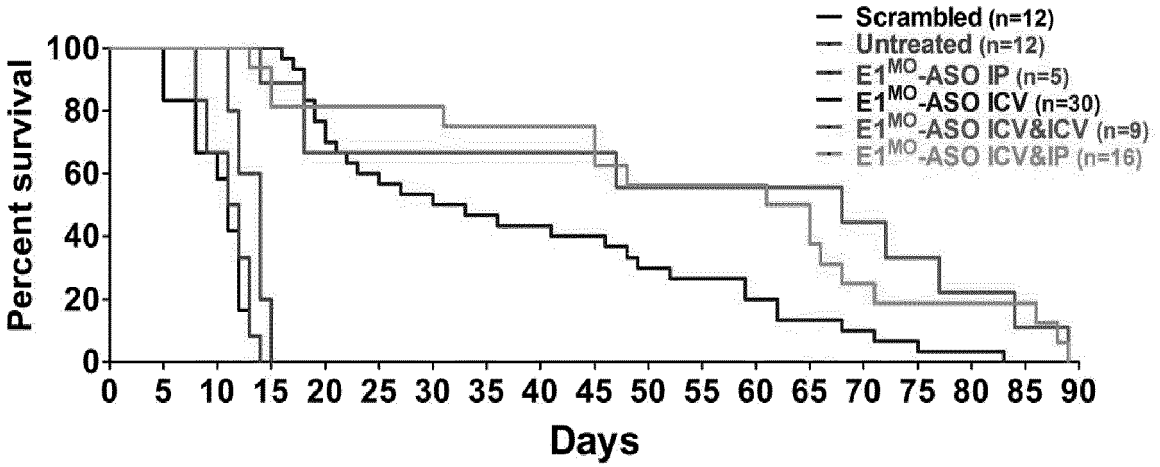


FIG. 16a

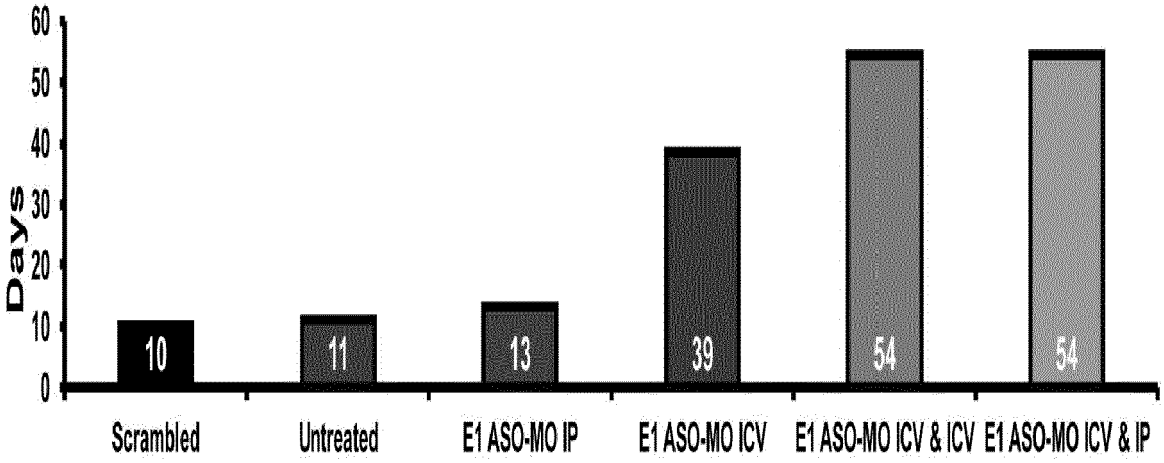


FIG. 16b

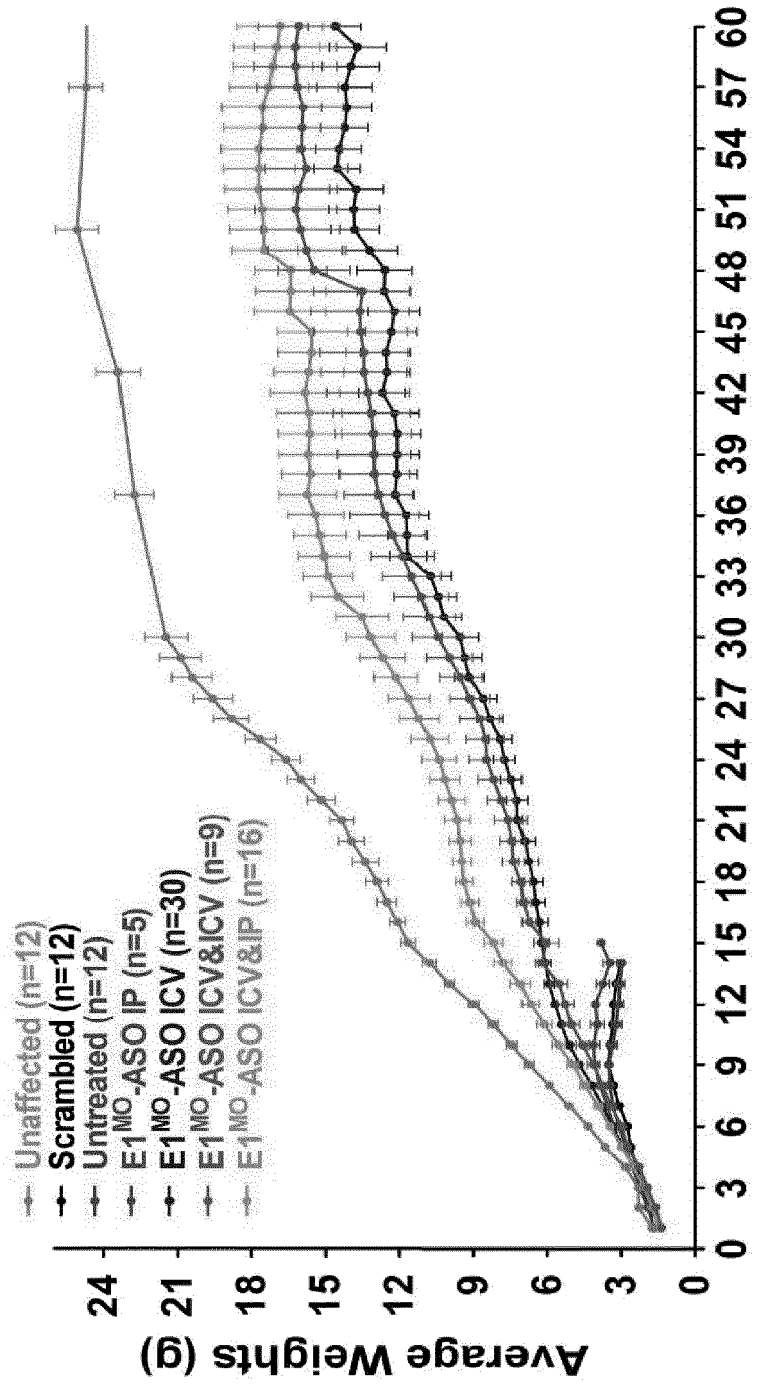

Unaffected (n=12)
Scrambled (n=12)
Untreated (n=12)
E1MO-ASO IP (n=5)
E1MO-ASO ICV (n=30)
E1MO-ASO ICV&ICV (n=9)
E1MO-ASO ICV&IP (n=16)
Average Weights (g)
0
3
6
9
12
15
18
21
24
Days
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60

FIG. 17

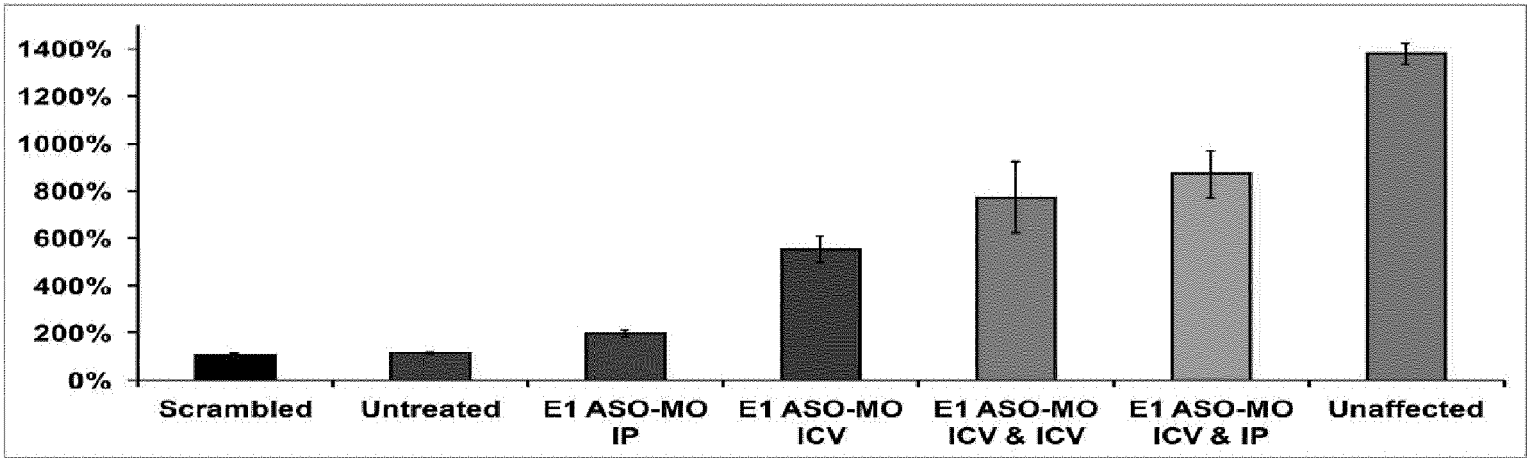


FIG. 18a

Treatment	Percent Weight Gain	P value Scrambled	P value Untreated	P value $E1^{MO}$-ASO IP	P value $E1^{MO}$-ASO ICV	P value $E1^{MO}$-ASO ICV&ICV	P value $E1^{MO}$-ASO ICV&IP	P value Unaffected
Scrambled	104%							
Untreated	115%	0.409080						
$E1^{MO}$- ASO IP	197%	0.000092	0.000011					
$E1^{MO}$- ASO ICV	551%	0.000011	0.000015	0.015203				
$E1^{MO}$- ASO ICV & ICV	774%	0.000065	0.000076	0.017202	0.092262			
$E1^{MO}$- ASO ICV & IP	874%	0.000001	0.000001	0.001456	0.003437	0.575766		
Unaffected	1380%	0.000000	0.000000	0.000000	0.000000	0.000381	0.000312	

FIG. 18b

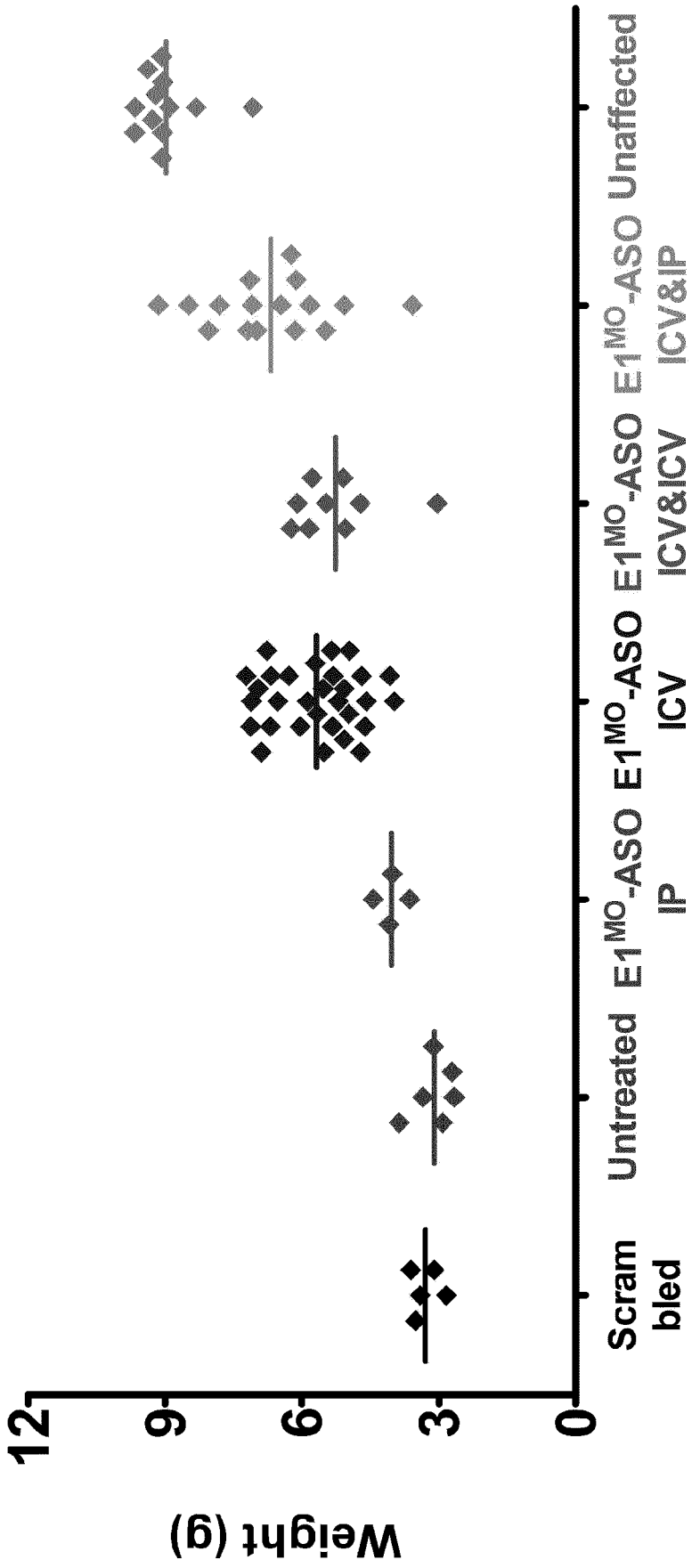

Weight (g)
12
9
6
3
0
Scram bled
Untreated
E1MO-ASO IP
E1MO-ASO ICV
E1MO-ASO ICV&ICV
E1MO-ASO ICV&IP
Unaffected

FIG. 19

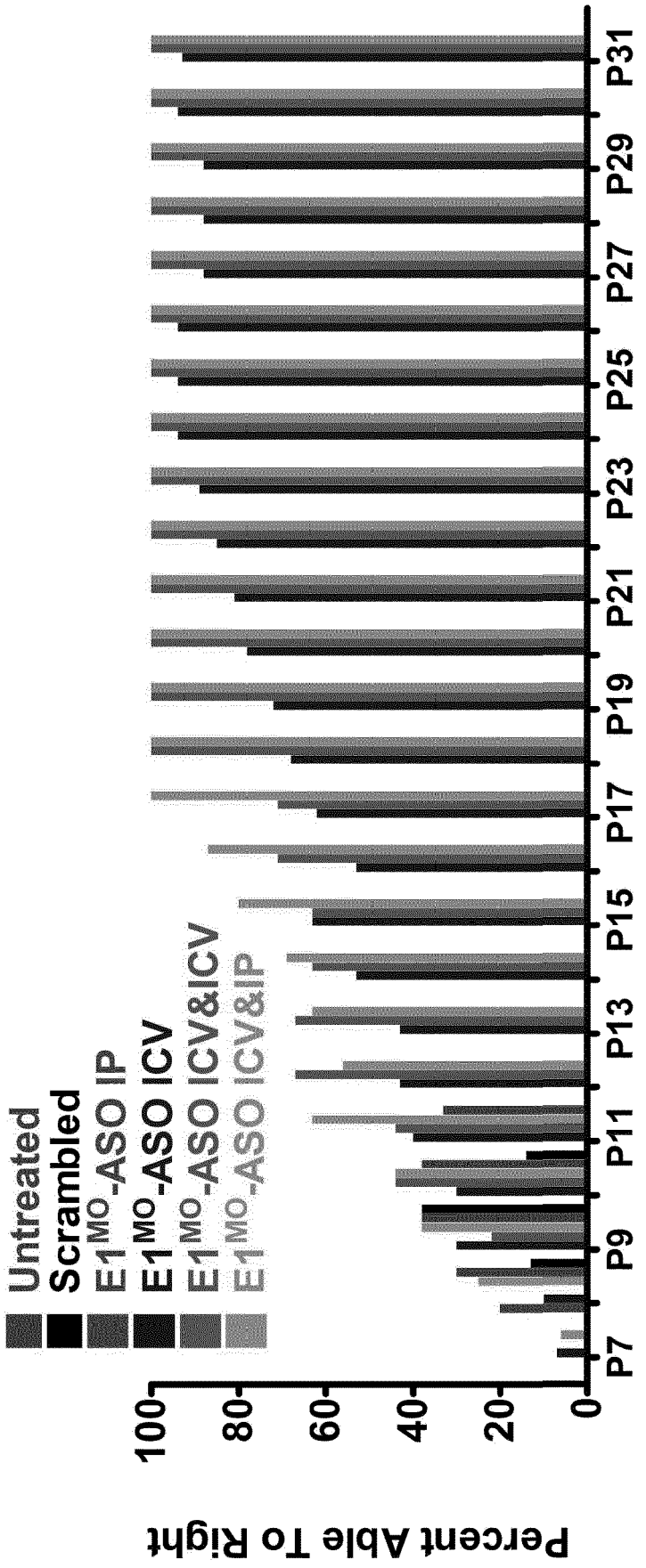

Untreated
Scrambled
E1MO-ASO IP
E1MO-ASO ICV
E1MO-ASO ICV&ICV
E1MO-ASO ICV&IP
Percent Able To Right
0
20
40
60
80
100
P7
P9
P11
P13
P15
P17
P19
P21
P23
P25
P27
P29
P31

FIG. 20

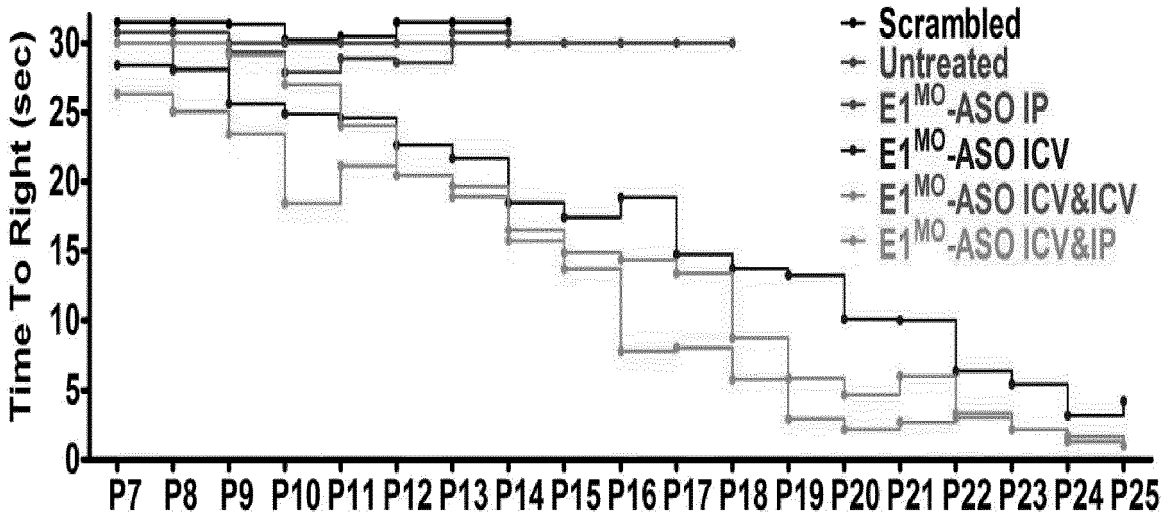


FIG. 21a

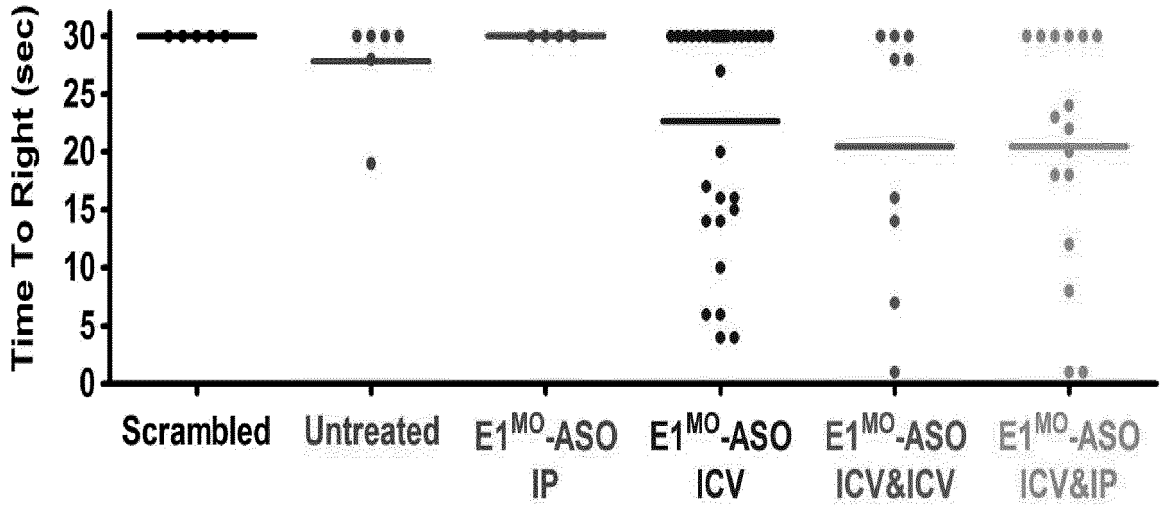


FIG. 21b

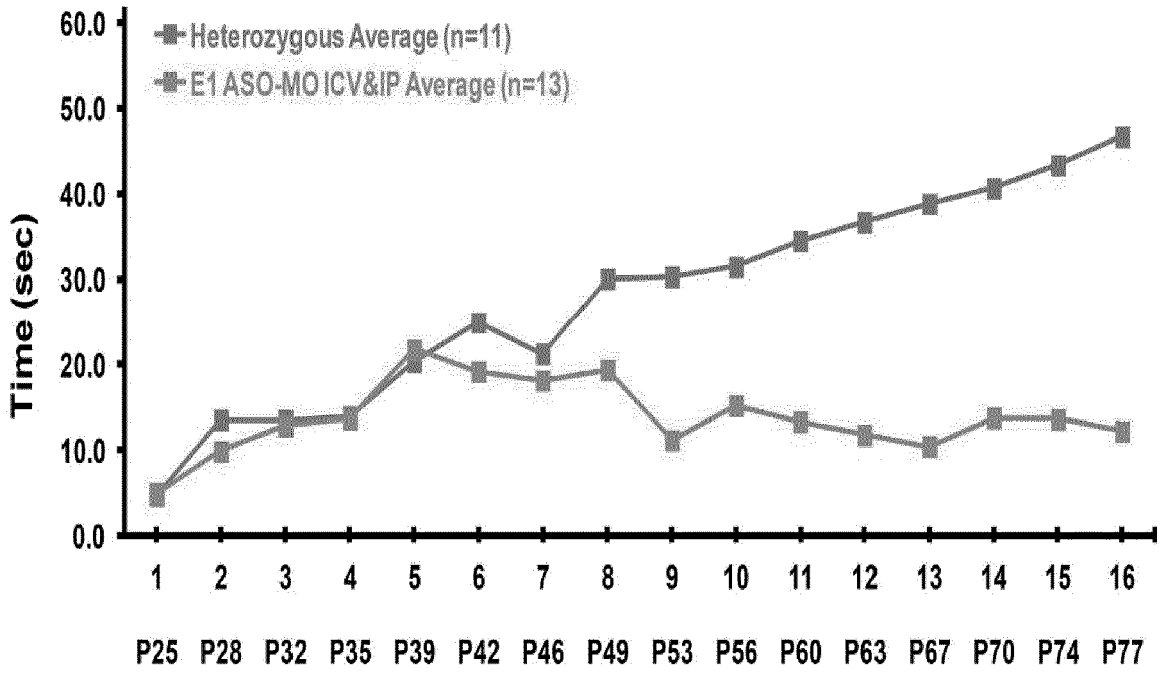

Heterozygous Average (n=11)
E1 ASO-MO ICV&IP Average (n=13)
Time (sec)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
P25 P28 P32 P35 P39 P42 P46 P49 P53 P56 P60 P63 P67 P70 P74 P77
Number of Trials/Days

FIG. 22

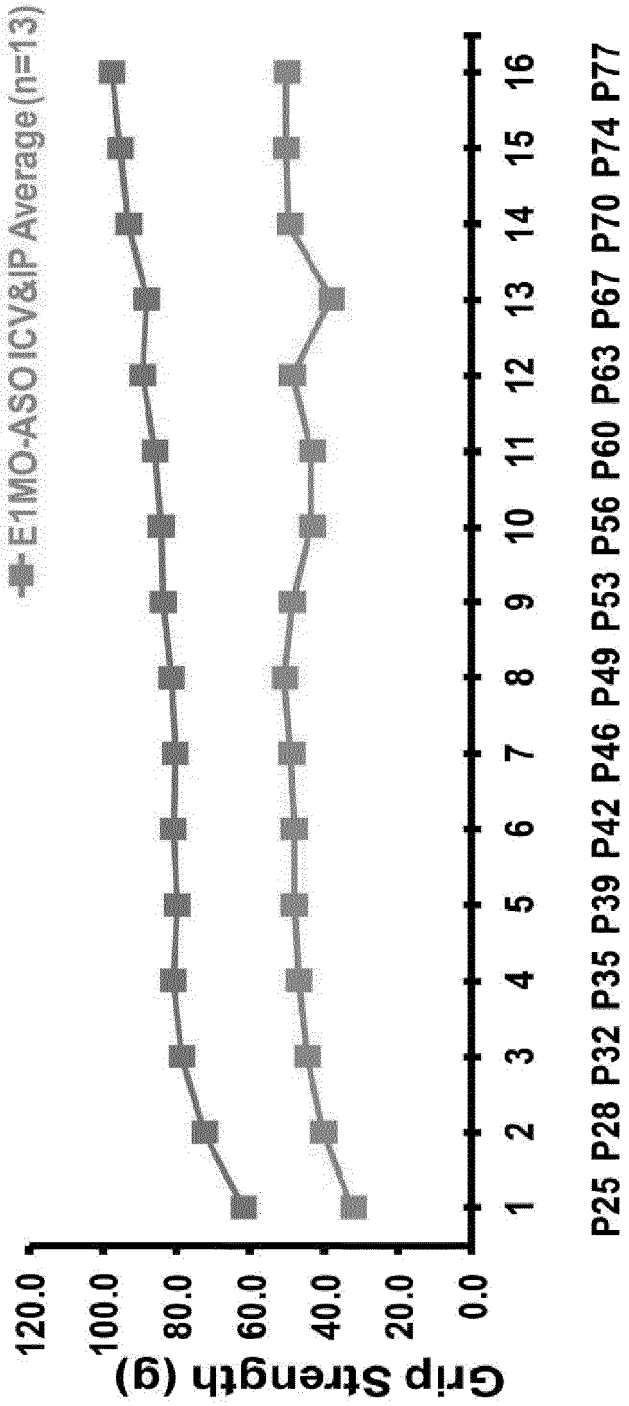

Heterozygous Average (n=11)
E1MO-ASO ICV&IP Average (n=13)
Grip Strength (g)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
P25 P28 P32 P35 P39 P42 P46 P49 P53 P56 P60 P63 P67 P70 P74 P77
Number of Trials/Days

FIG. 23

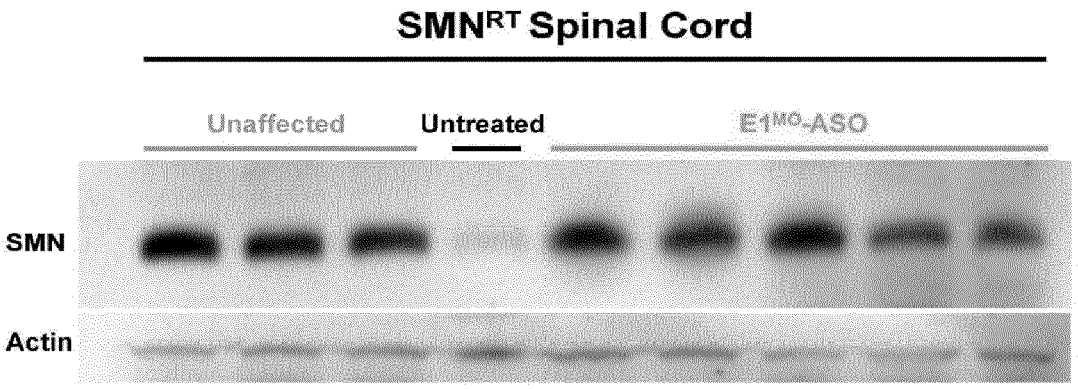


FIG. 24a

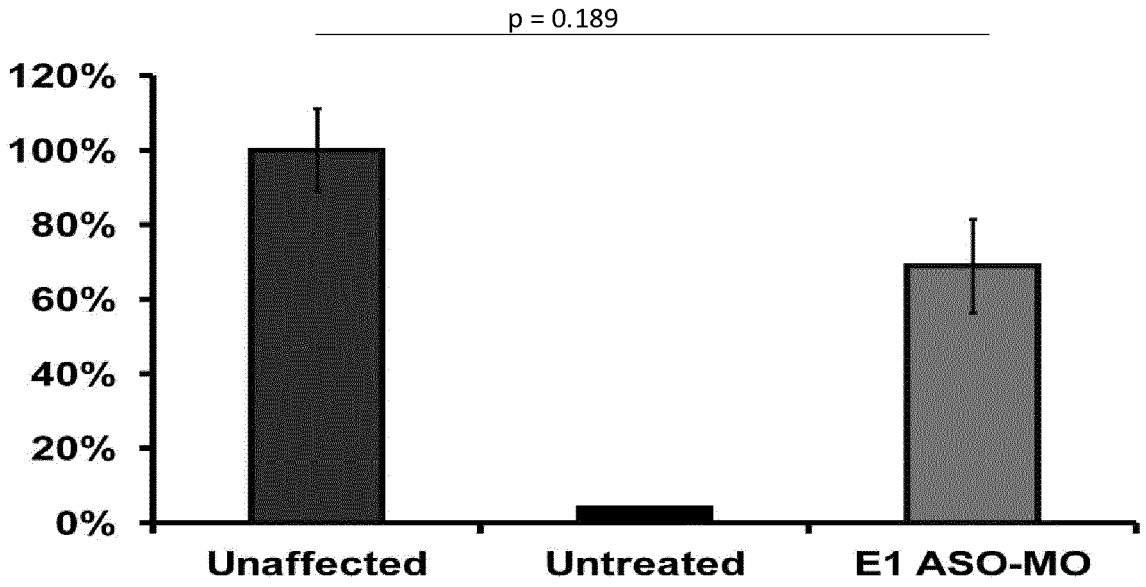


FIG. 24b

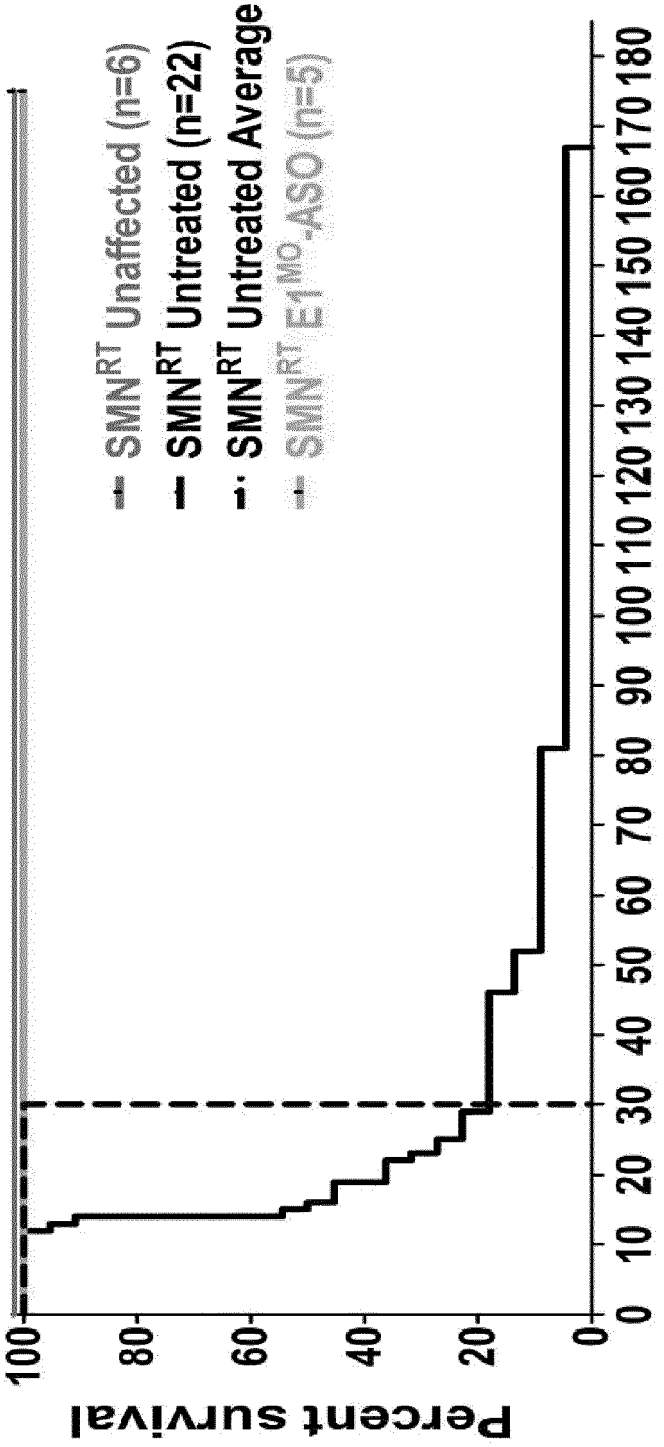

SMN^RT Unaffected (n=6)
SMN^RT Untreated (n=22)
SMN^RT Untreated Average
SMN^RT E1^MO-ASO (n=5)
Percent survival
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Days

FIG. 25

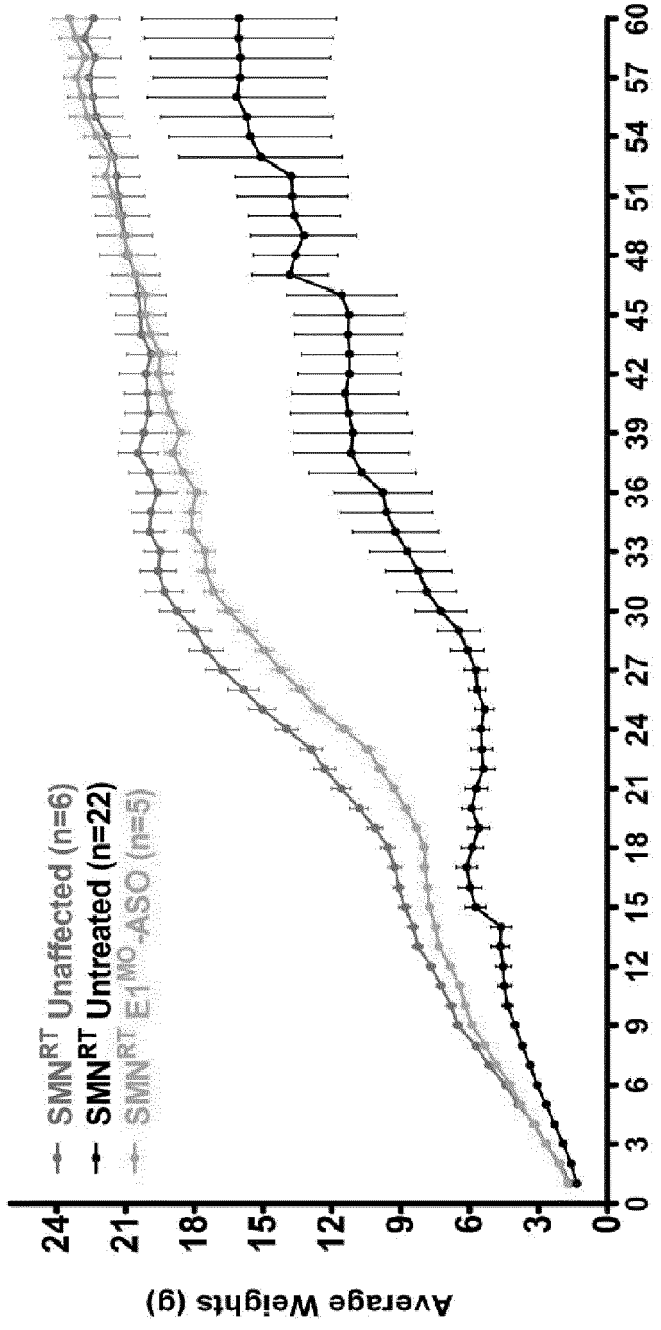

SMN^RT Unaffected (n=6)
SMN^RT Untreated (n=22)
SMN^RT E1^MO-ASO (n=5)
Average Weights (g)
0
3
6
9
12
15
18
21
24
Days
0
3
6
9
12
15
18
21
24
27
30
33
36
39
42
45
48
51
54
57
60

FIG. 26

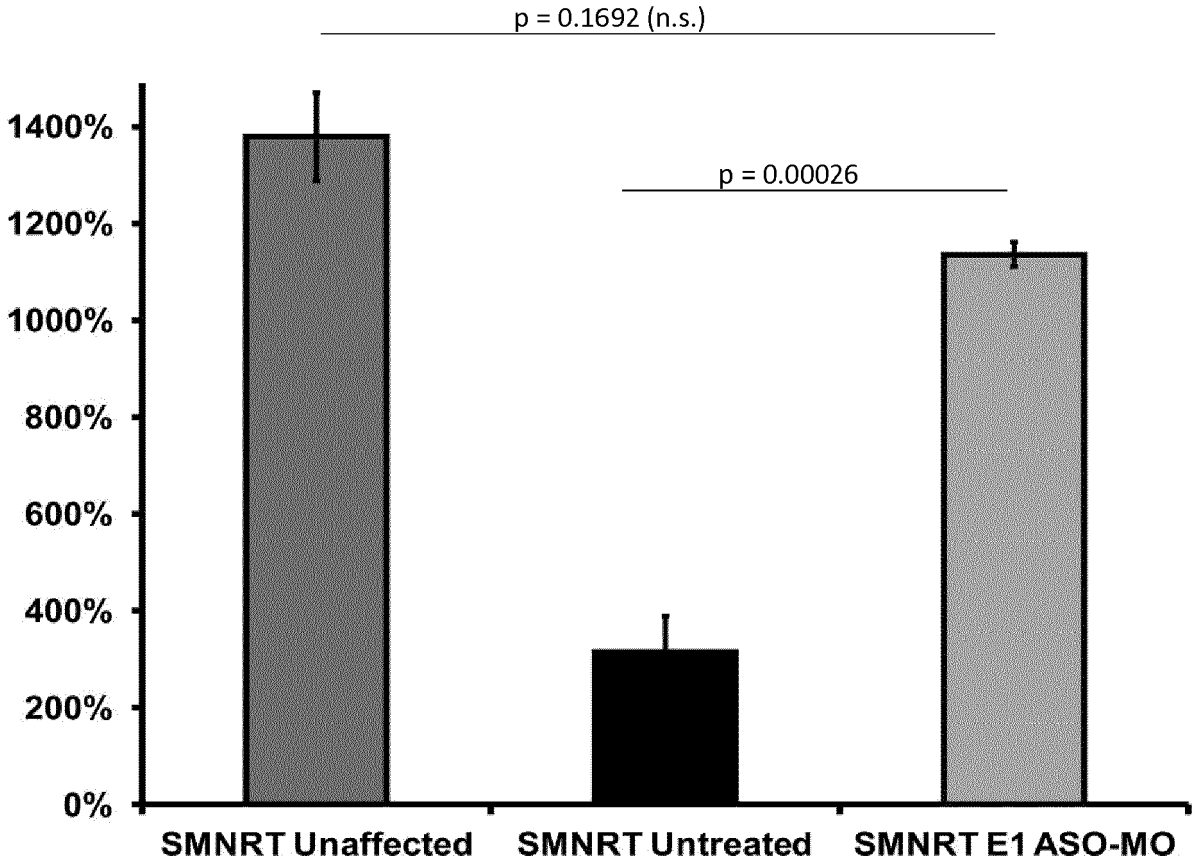

p = 0.1692 (n.s.)
p = 0.00026
1400%
1200%
1000%
800%
600%
400%
200%
0%
SMNRT Unaffected
SMNRT Untreated
SMNRT E1 ASO-MO

FIG. 27

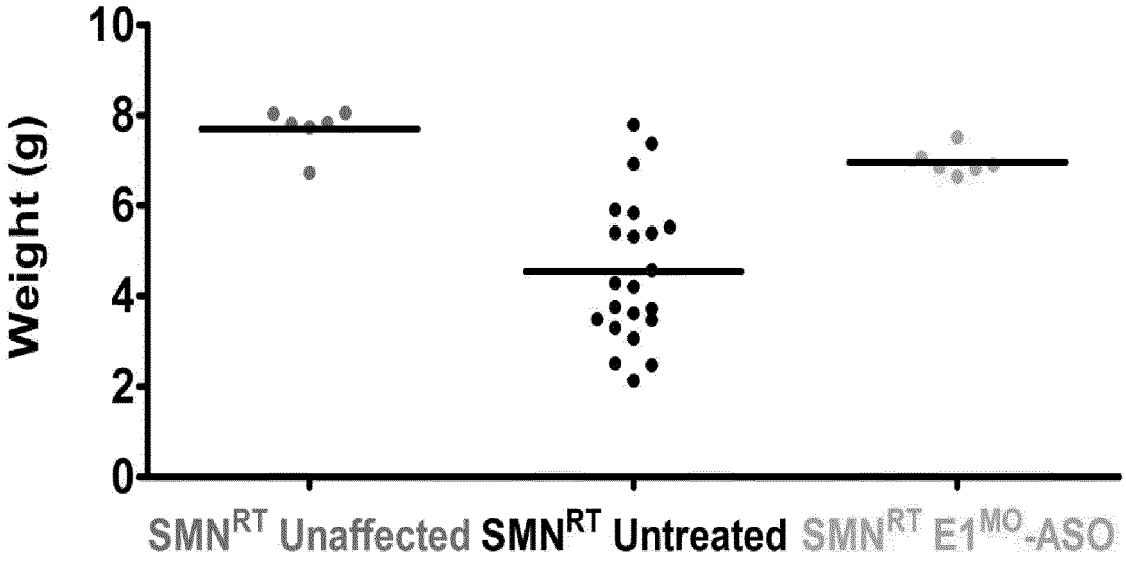

Weight (g)
10
8
6
4
2
0
SMN^RT Unaffected
SMN^RT Untreated
SMN^RT E1^MO-ASO

FIG. 28

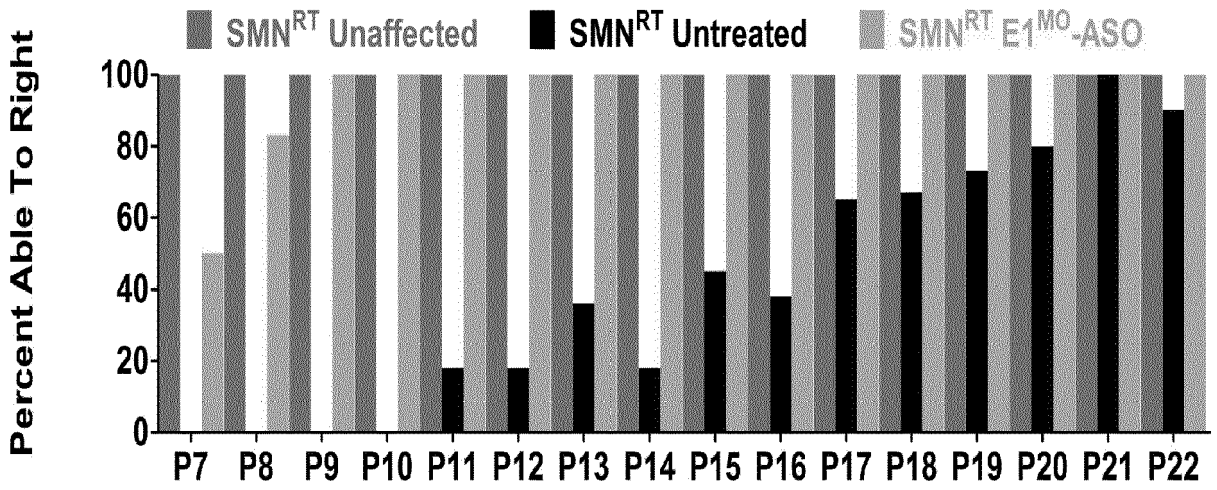

SMN^RT Unaffected
SMN^RT Untreated
SMN^RT E1^MO-ASO
Percent Able To Right
100
80
60
40
20
0
P7
P8
P9
P10
P11
P12
P13
P14
P15
P16
P17
P18
P19
P20
P21
P22

FIG. 29

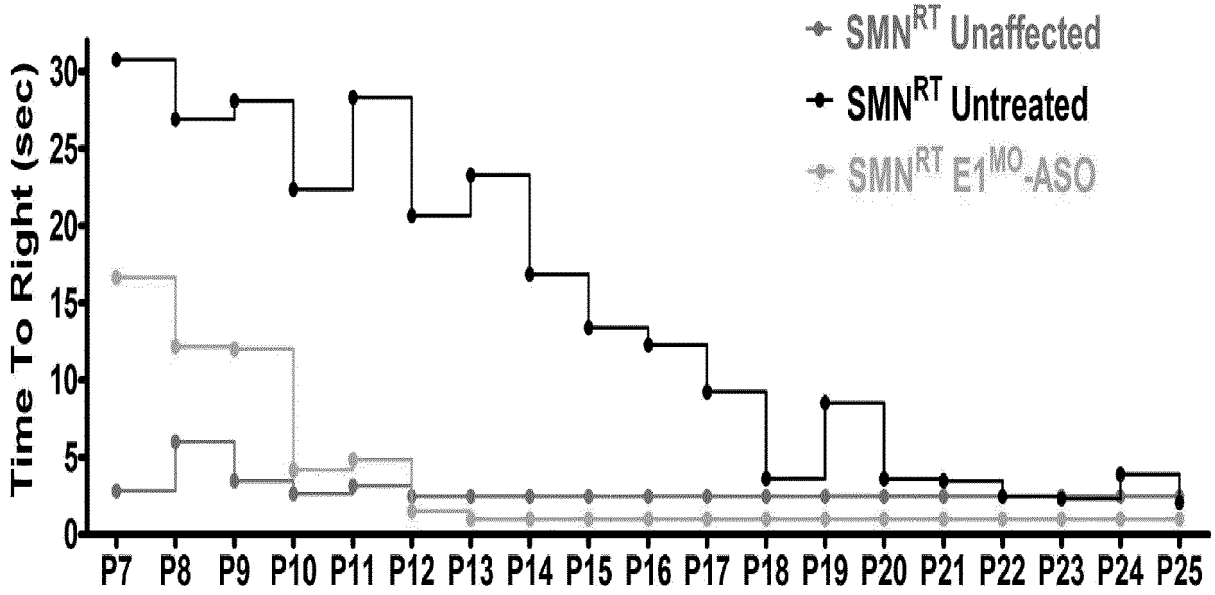

SMN^RT Unaffected
SMN^RT Untreated
SMN^RT E1^MO-ASO
Time To Right (sec)
0
5
10
15
20
25
30
P7
P8
P9
P10
P11
P12
P13
P14
P15
P16
P17
P18
P19
P20
P21
P22
P23
P24
P25

FIG. 30

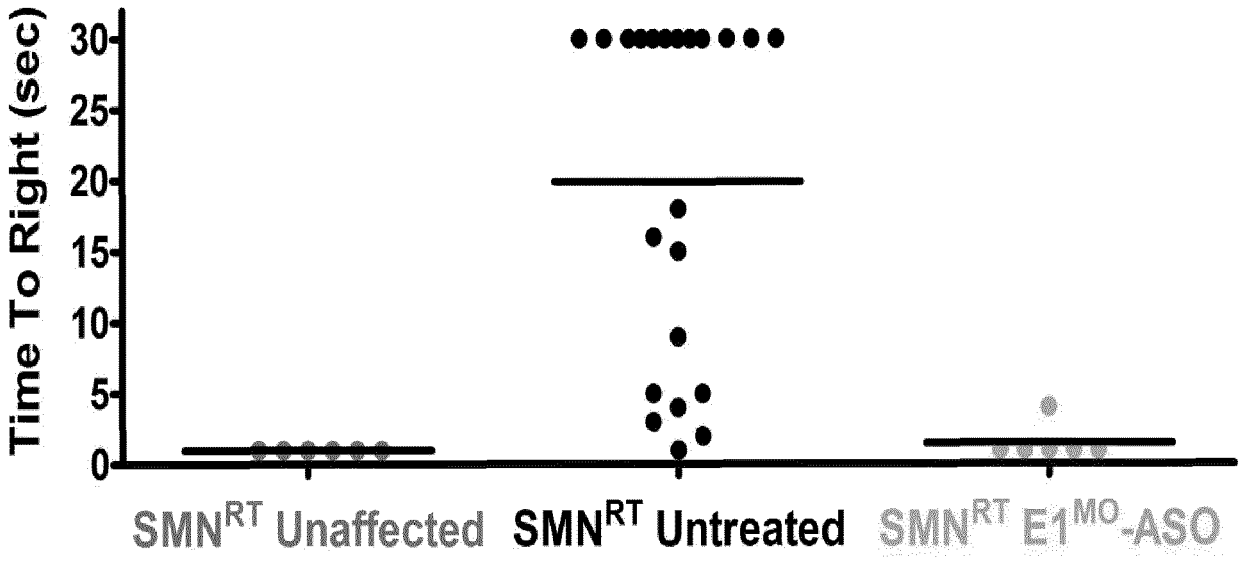

Time To Right (sec)
30
25
20
15
10
5
0
SMN^RT Unaffected
SMN^RT Untreated
SMN^RT E1^MO-ASO

FIG. 31

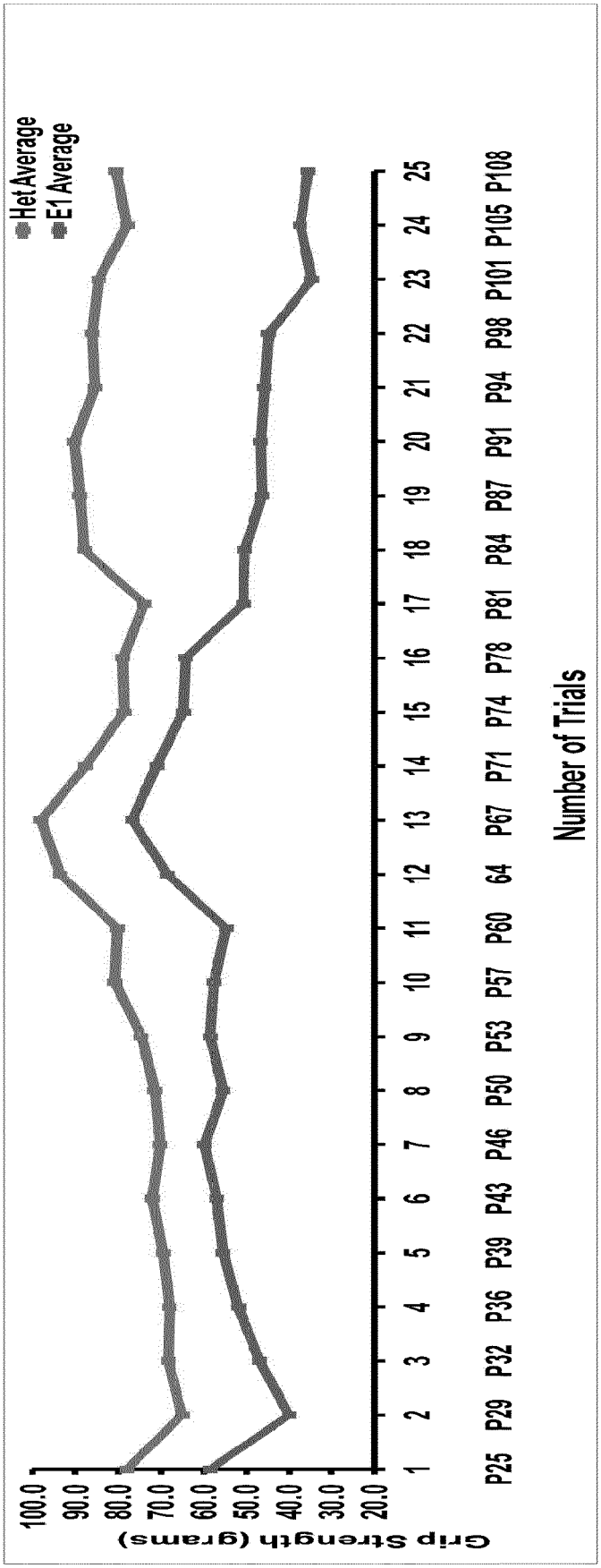

Het Average
E1 Average
Grip Strength (grams)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
P25 P29 P32 P36 P39 P43 P46 P50 P53 P57 P60 64 P67 P71 P74 P78 P81 P84 P87 P91 P94 P98 P101 P105 P108
Number of Trials

FIG. 32

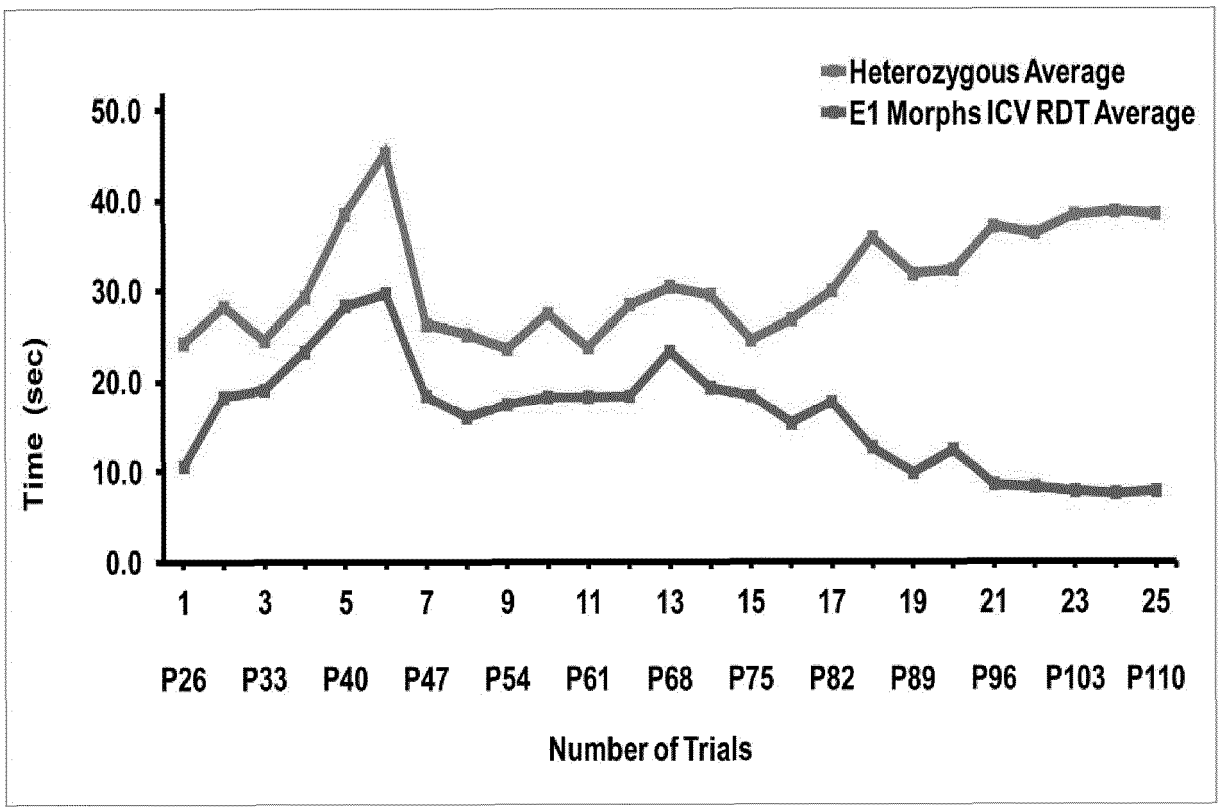

Heterozygous Average
E1 Morphs ICV RDT Average
50.0
40.0
30.0
20.0
10.0
0.0
Time (sec)
1
3
5
7
9
11
13
15
17
19
21
23
25
P26
P33
P40
P47
P54
P61
P68
P75
P82
P89
P96
P103
P110
Number of Trials

FIG. 33

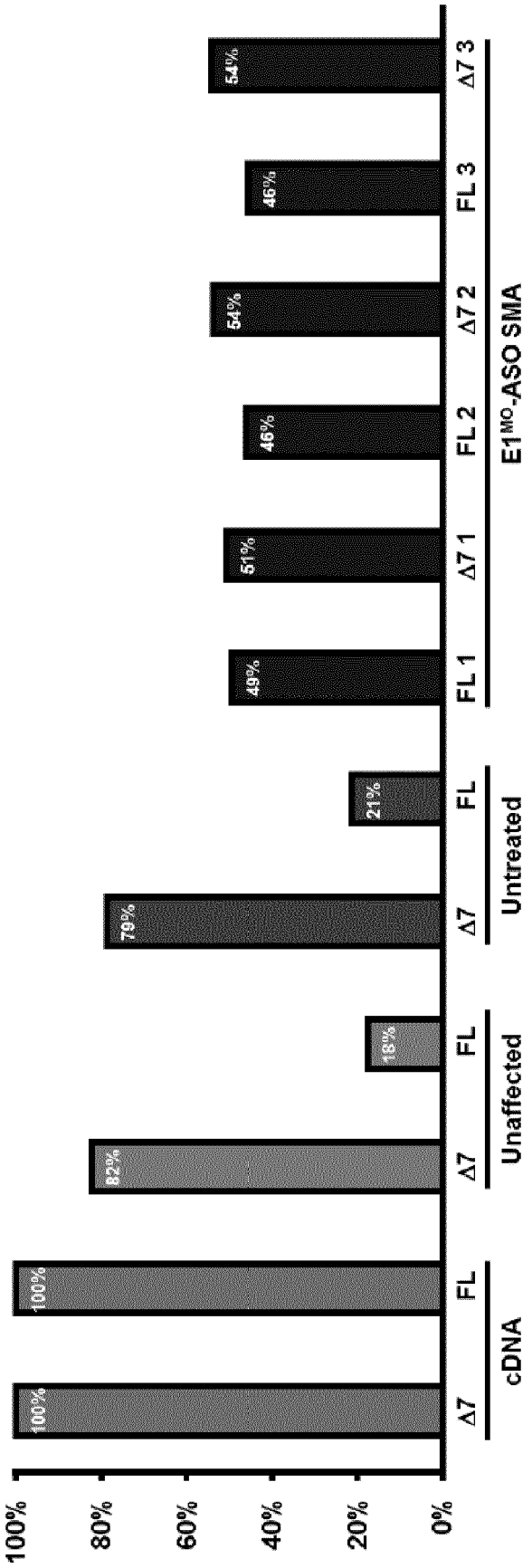

100%
80%
60%
40%
20%
0%
100%
100%
82%
18%
79%
21%
49%
51%
46%
54%
46%
54%
Δ7
FL
Δ7
FL
Δ7
FL
FL 1
Δ7 1
FL 2
Δ7 2
FL 3
Δ7 3
cDNA
Unaffected
Untreated
E1MO-ASO SMA

FIG. 34

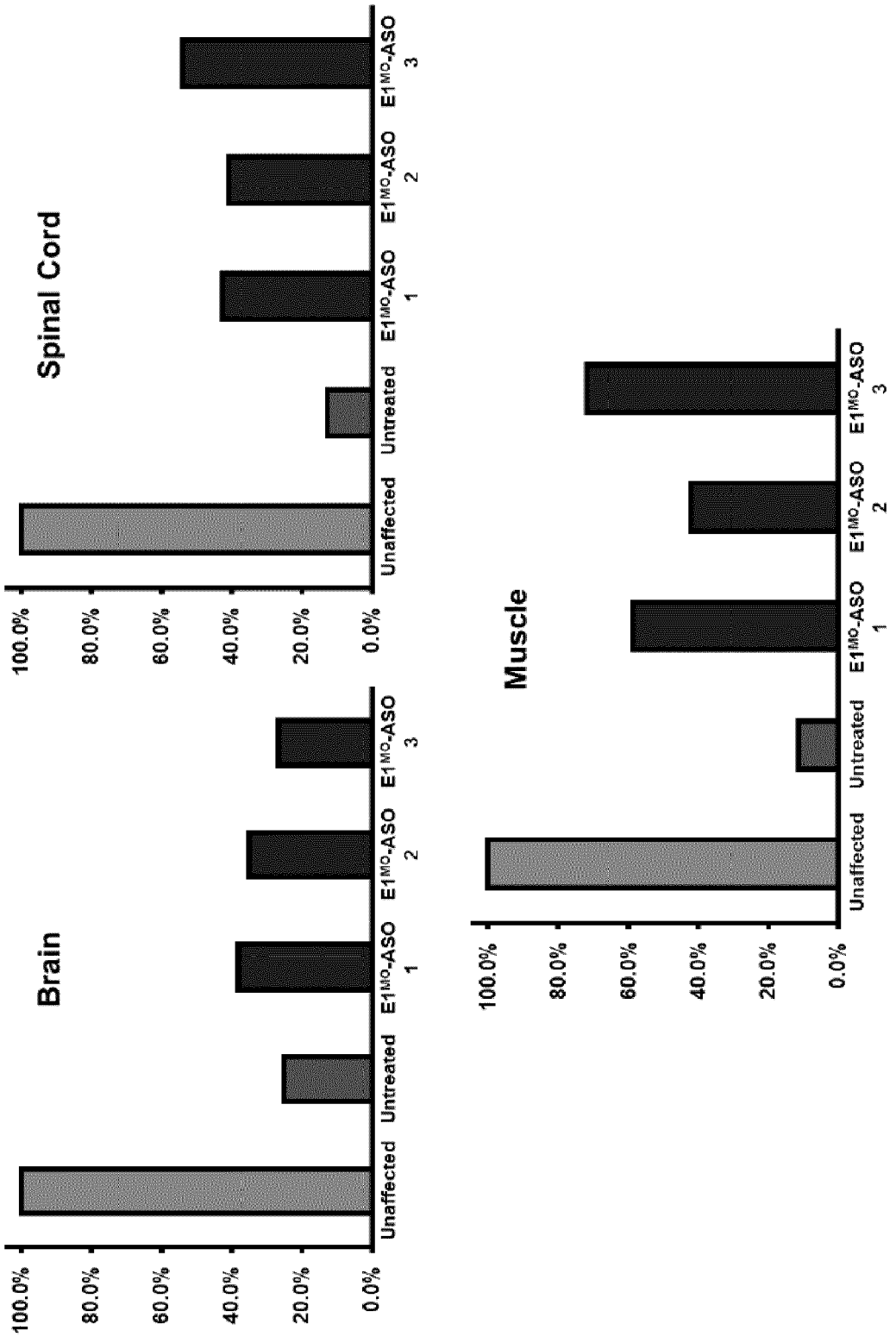

Brain
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
Unaffected
Untreated
E1MO-ASO 1
E1MO-ASO 2
E1MO-ASO 3
Spinal Cord
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
Unaffected
Untreated
E1MO-ASO 1
E1MO-ASO 2
E1MO-ASO 3
Muscle
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
Unaffected
Untreated
E1MO-ASO 1
E1MO-ASO 2
E1MO-ASO 3

FIG. 35

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 19 16 3664

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	Baughan ET AL: "BAUGHAN, TRAVIS.: 'Gene Therapy in Spinal Muscular Atrophy: RNA-Based Strategies to Modulate the Pre-mRNA Splicing of Survival Motor Neuron'", DISSERTATION, 1 December 2008 (2008-12-01), XP055290503, Retrieved from the Internet: URL:https://mospace.umsystem.edu/xmlui/bitstream/handle/10355/6686/public.pdf?sequence=1&isAllowed=y [retrieved on 2016-07-22]	1-7, 9-13,15	INV. A61K31/712
Y	* pages 112, 122; figures 1, 2a * * pages 119-120 *	8,14	
X	ERKAN Y OSMAN ET AL: "Bifunctional RNAs Targeting the Intronic Splicing Silencer N1 Increase SMN Levels and Reduce Disease Severity in an Animal Model of Spinal Muscular Atrophy", MOLECULAR THERAPY, vol. 20, no. 1, 1 January 2012 (2012-01-01), pages 119-126, XP055051390, ISSN: 1525-0016, DOI: 10.1038/mt.2011.232	1-7, 9-13,15	TECHNICAL FIELDS SEARCHED (IPC) C12N
Y	* the whole document *	8,14	
X	T. D. BAUGHAN ET AL: "Delivery of bifunctional RNAs that target an intronic repressor and increase SMN levels in an animal model of spinal muscular atrophy", HUMAN MOLECULAR GENETICS, vol. 18, no. 9, 1 January 2009 (2009-01-01), pages 1600-1611, XP055051447, ISSN: 0964-6906, DOI: 10.1093/hmg/ddp076	1-7, 9-13,15	
Y	* the whole document *	8,14	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	9 July 2019	Franz, Cerstin

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 19 16 3664

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	TW 201 007 167 A (LIN TA-CHIN [TW]) 16 February 2010 (2010-02-16)	2	
A	* sequence AZG72615 *	1,3-15	
X	US 5 882 868 A (FUNANAGE VICKY LINN [US] ET AL) 16 March 1999 (1999-03-16)	2	
A	* sequences AAX02904, AAX2904, AAX02903 *	1,3-15	
X	US 2011/118145 A1 (AKMAEV VIATCHESLAV R [US] ET AL) 19 May 2011 (2011-05-19)	2	
A	* sequence AZI43921 *	1,3-15	
X	EP 2 499 262 A1 (ESOTERIX GENETIC LAB LLC [US]) 19 September 2012 (2012-09-19)	2	
A	* sequence AZI43921 *	1,3-15	
Y	WO 2010/120820 A1 (ISIS PHARMACEUTICALS INC [US]; COLD SPRING HARBOR LAB [US]; KRAINER AD) 21 October 2010 (2010-10-21)	8,14	
A	* paragraph [0100] *	1-7, 9-13,15	TECHNICAL FIELDS SEARCHED (IPC)
Y	WO 2010/148249 A1 (ISIS PHARMACEUTICALS INC [US]; GENZYME CORP [US]; COLD SPRING HARBOR L) 23 December 2010 (2010-12-23)	8,14	
A	* page 14, lines 11-15 *	1-7, 9-13,15	
A	AGA LEWELT ET AL: "New Therapeutic Approaches to Spinal Muscular Atrophy", CURRENT NEUROLOGY AND NEUROSCIENCE REPORTS, CURRENT SCIENCE, PHILADELPHIA, PA, US, vol. 12, no. 1, 2 December 2011 (2011-12-02), pages 42-53, XP035000591, ISSN: 1534-6293, DOI: 10.1007/S11910-011-0240-9 * the whole document *	1-15	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	9 July 2019	Franz, Cerstin

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 19 16 3664

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

09-07-2019

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
TW 201007167 A	16-02-2010	NONE	
US 5882868 A	16-03-1999	NONE	
US 2011118145 A1	19-05-2011	CA 2777549 A1	19-05-2011
		CN 102597272 A	18-07-2012
		EP 2499262 A1	19-09-2012
		JP 2013510580 A	28-03-2013
		US 2011118145 A1	19-05-2011
		US 2016319339 A1	03-11-2016
		WO 2011060240 A1	19-05-2011
EP 2499262 A1	19-09-2012	CA 2777549 A1	19-05-2011
		CN 102597272 A	18-07-2012
		EP 2499262 A1	19-09-2012
		JP 2013510580 A	28-03-2013
		US 2011118145 A1	19-05-2011
		US 2016319339 A1	03-11-2016
		WO 2011060240 A1	19-05-2011
WO 2010120820 A1	21-10-2010	US 2012149757 A1	14-06-2012
		WO 2010120820 A1	21-10-2010
WO 2010148249 A1	23-12-2010	AU 2010262862 A1	19-01-2012
		AU 2016200344 A1	11-02-2016
		CA 2765396 A1	23-12-2010
		CN 102665731 A	12-09-2012
		CN 106983768 A	28-07-2017
		DK 3305302 T3	19-11-2018
		EP 2442816 A1	25-04-2012
		EP 3305302 A1	11-04-2018
		EP 3449926 A1	06-03-2019
		ES 2699827 T3	13-02-2019
		HR P20181712 T1	28-12-2018
		HU E039741 T2	28-01-2019
		IL 217014 A	21-04-2016
		IL 245096 A	30-04-2018
		JP 5707396 B2	30-04-2015
		JP 6064139 B2	25-01-2017
		JP 6370860 B2	08-08-2018
		JP 2012530715 A	06-12-2012
		JP 2015131828 A	23-07-2015
		JP 2017061497 A	30-03-2017
		JP 2018184423 A	22-11-2018
		KR 20120093138 A	22-08-2012
		KR 20170054537 A	17-05-2017

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 19 16 3664

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

09-07-2019

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		KR 20190017063 A	19-02-2019
		KR 20190037378 A	05-04-2019
		LT 3305302 T	10-12-2018
		MX 361732 B	14-12-2018
		NZ 597071 A	30-05-2014
		NZ 624712 A	30-10-2015
		PL 3305302 T3	28-02-2019
		RU 2012101491 A	27-07-2013
		RU 2015139553 A	25-12-2018
		SI 3305302 T1	31-12-2018
		TR 201816256 T4	21-11-2018
		US 2012190728 A1	26-07-2012
		US 2017015995 A1	19-01-2017
		US 2019030058 A1	31-01-2019
		WO 2010148249 A1	23-12-2010

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 61853820 A **[0001]**
- US 8110560 B2, Singh **[0006]**
- US 8110560 B **[0006]**